SIMULTANEOUS DETERMINATION AND VALIDATION OF OFLOXACIN AND ORNIDAZOLE IN COMBINED DOSAGE PHARMACEUTICAL FORMULATION BY RP-HPLC METHOD

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Dedicated
To
My Teachers & Friends

CONTENTS

LIST OF ABBREVIATIONS

HPLC	-	High Performance Thin Layer Chromatography
ICH	-	International Conference on Harmonization
g	-	Grams
ml	-	Mililitre
nm	-	Nanometer
pH	-	Negative Logarithm of Hydrogen Ion
RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
Rt	-	Retention Time
S.D	-	Standard Deviation
v/v	-	Volume/Volume
%	-	Percentage
%RSD	-	Percentage Relative Standard Deviation
µ	-	Micron
µl	-	Microlitre
µg/ml	-	Microgram per Mililiter
USP	-	United States of Pharmacopoeia
˚C	-	Degree centigrade
IP	-	Indian Pharmacopoeia
l	-	Litre
LOD	-	Limit Of Detection
LOQ	-	Limit Of Quantification
λmax	-	Lamda max, Absorption Maximum

USP	-	United states of Pharmacopoeia
UV	-	Ultraviolet
ng	-	Nanogram
r	-	Regression Coefficient
µL min-1	-	Microlitre Per Minute
mg mL-1	-	Milligram Per Millilitre
µg mL-1	-	Microgram Per Millilitre
Rf	-	Retardation Factor
˚C	-	Degree centigrade

Chapter – 1

Introduction

1. INTRODUCTION

Analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical Analysis derives its principles from various branches like chemistry, physics, microbiology etc., pharmaceutical analytical techniques are applied mainly in two areas, viz quantitative analysis and qualitative analysis, although there are several other applications.

Today, more than 80% of all analytical chromatographic separations are performed using reversed-phase adsorbents which have shown higher versatility compared to normal-phase chromatography adsorbents. Reversed-phase adsorbents have found their use in a wide range of applications such as process purification, isolation of active bio molecules, analytical separation of drugs and metabolites as well as extraction of various contaminants in environmental samples.

However, Reversed-Phase Chromatography includes a large number of different phases that differ significantly in both chemical and physical properties which will have a significant impact on their chromatographic behaviours. Thus, one chromatographer will have to consider all of the following stationary phase properties while gathering information on the sample to be analyzed or purified such as

1. Hydrophobicity
2. Particle and Pore Diameter
3. Bonding Chemistry (Surface Coverage, Polymeric vs Monomeric)
4. Particle Geometry (Irregular vs Spherical)

5. Silica Purity
6. Analyte pKa
7. Molecular Weight
8. Sample Matrix (pH, salts, concentration, interference)Analytical techniques

Drugs and pharmaceuticals are chemicals or like substances, which are of organic, inorganic or other origin. Whatever may be the origin, we use some property of the medicinal agent to measure them qualitatively or quantitatively. Pharmaceutical analytical techniques, which are being used, can be categorized as follows.

Spectroscopy:

The light absorption (or) emission characteristics of drugs are measured in UV-Visible, IR, NMR, ESR, fluorescence and mass spectroscopy.

Chromatography:

It is a separation technique where the affinity or partition co-efficient differences between the drugs are recorded with TLC, HPLC, GC, Column and paper chromatography.

Electro analytical techniques:

It is measured based on the electrochemical property of drugs and classified as potentiometry, conductometry, polarography, amperometry and paper electrophoresis.

Radioactive methods:

In this method, radiation intensity from a radioactive substance or an induced radioactive substance arising from exposure of the sample to a neutron source is measured using scintillation counters, e.g., Radio Immuno Assay. **(Beckett A.H, 1997)**

Physical methods:

The physical characteristics of drugs are measured such as DSC, DTA, TGA and TMA.

Titrimetric methods:

It is based on the reaction between titrant and titrate and the end point detected by the addition of a suitable indicator reagent. It is classified in to several types based on the reaction which is non aqueous, redox, diazotization and complexometric titrations.

X-ray methods:

In this, x-ray is used to identify certain emission peaks, which are characteristics of elements contained in the target. The wavelength of the peak can be related to the atomic numbers of the elements producing them.

Hyphenated techniques:

It is the combination of two or more methods where separation and identification is possible by using coupled instruments such as GC-MS and LC-MS. **(ICH Topic Q2A. Text Nov'1996)**

Classification of chromatography techniques:

A. Based on modes of chromatography:

- Normal phase chromatography
- Reverse phase chromatography

B. Based on principle of separation:

- Adsorption chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Affinity chromatography
- Chiral phase chromatography

C. Based on elution:

- Isocratic separation
- Gradient separation

D. Based on the scale of operation:

- Analytical HPLC
- Preparative HPLC

Normal Phase:

The hydrophilic molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of a particle if that surface is also hydrophilic. Increasing the polarity of the mobile phase will subsequently decrease the adsorption and ultimately cause the sample molecules to exit the column. This mechanism is called Normal Phase Chromatography. It is a very powerful technique that often requires non-polar solvents. Due to safety and environmental concerns this mode is used mostly as an analytical technique and not for process applications. **(Chatwal.R., et.al., 2000)**

Reverse Phase:

The reverse phase chromatography, results from the adsorption of hydrophobic molecules on to a hydrophobic solid support in a polar mobile phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the solid support resulting in de-sorption. The more hydrophobic the molecule the more time it will spend on the solid support and the higher the concentration of organic solvent that is required to promote de-sorption. **(Sharma B.K 1999).**

Reversed phase chromatography (RPC) is the most popular separation technique at analytical scale, because:

- RPC applies to a very wide range of molecules including charged and polar molecules,

- RPC allows precise control of variables such as organic solvent type and concentration, pH, and temperature,
- RPC columns are efficient and stable,
- RPC is a robust technique.

High Performance Liquid Chromatography:

The analytical technique of High Performance of Liquid Chromatography (HPLC) is used extensively throughout the pharmaceutical industry. It is used to provide the information on the composition of drug related samples. The information obtained may be quantitative, indicating what compounds are present in sample or quantitative, providing the actual amounts of compounds in the sample. HPLC is used at all the different stages in creation of new drug and also is used routinely during drug manufacture. The aim of analysis will depend on the both the nature of the sample and the stage of the development. HPLC is a chromatographic technique; therefore it is necessary to have basic understanding of chromatography to understand how it works. **(Donald Willings A 2011).**

Chromatography:

A Russian botanist, Mi Khail Tswett is credited with the first use of chromatography in 1906 when he separated plant pigments such as chlorophylls and xanthophylls. He passed them through a glass column packed with calcium carbonate. These pigments are coloured and thus the technique was named using the Greek terms, 'chroma' meaning 'colour', and 'graphein' meaning ' to write1. This explains

why the name seemingly bears little relation to the use of technique today. **(Beckett 2002, Galen W.E 1977)**.

Chromatography is a technique which separates components in a mixture due to the different time taken for each component to travel through a stationary phase when carried through it by a mobile phase.

The possible mixtures of phases give rise to the types of chromatography listed in below table.

Types of Chromatography:

TYPE OF CHROMATOGRAPHYMOBILE PHASE STATIONARY PHASE

Type of Chromatography	Mobile Phase	Stationary Phase
Gas chromatography	Gas	Solid/liquid
Liquid chromatography	Liquid	Solid/liquid
Supercritical-fluid chromatography	Supercritical fluid	Solid/liquid

The stationary phase is fixed in place either in column (a hallow tube made out of a suitable material, e.g. Glass) or on a planar surface and the mobile phase moves over or through the stationary phase carrying with it the sample of interest. In practice the stationary phase can be a solid, a liquid adsorbed on a solid or an organic species (e.g. A cl8 alkyl chain) bonded to a solid surface. In gas chromatography, supercritical-fluid chromatography the stationary phase is fixed in place in a column. In liquid chromatography the stationary phase may be fixed in either column or on a planar surface. In HPLC a column is used. The name given to liquid chromatography

on a planar surface is Thin Layer Chromatography (TLC). **(Chatwal R and Anand 2000).**

The Stationary phase:

It is the combination of suitable stationary phase and mobile phase that enables the separation of mixture and thus analysis of the component in the mixture. HPLC is characterized by the use of very small particles of stationary phase which are fixed in place in a HPLC column, often made of a material such as stainless steel. **(ICH Topic Q2B, Nov'1996)**

Normal phase and Reverse phase HPLC:

In order to describe the different stationary phases available for HPLC it is necessary to explain the concept of normal and reverse phase HPLC. These types of HPLC vary due to the polarity of the stationary phase and mobile phase in each as shown in table below.

	Stationary phase	**Stationary Phase**
Normal phase	Polar	Non- polar
Reversed phase	Non-polar	Polar

Normal phase HPLC:

In a mixture of components to be separated those analytes which are relatively more polar will be retained by the polar stationary phase longer than those analytes which are relatively less polar. Therefore the least polar component will elute first.

The attractive forces which exist are mostly dipole-dipole and hydrogen bonding (polar) interactions. **(Indian Pharmacopeia, 2014)**

Reverse phase HPLC:

In a mixture of components to be separated those analytes which are relatively less polar will be retained by the non-polar stationary phase longer than those analytes which are relatively more polar. Therefore the most polar component will elute first. The attractive forces which exist are mainly non-specific hypdrophobic interactions. The exact nature of these interactions is still under discussion.

Packing/Matrix of the HPLC column:

The most common packing material used in HPLC columns is silica. It is physically robust and chemically stable in virtually all solvents and at low pH (it begins to dissolve around pH7). The manufacturing technology for silica production has improved substantially since the early days of HPLC. Irregular shaped particles contaminated with the metal impurities have given way to spherical particles with low levels of impurities. This purer silica is known as type B silica and the less pure pure material is known as type A silica.

Other non-silica based packing's have been introduced in last few years. Among them have been polystyrene-de-vinyl benzene polymers, other polymers, graphitized carbon and hydroxyapatite.

The mobile phase:

The mobile phase for HPLC is the liquid phase which is continually flowing through the stationary phase and which carries the analyte through with it. The

composition of the mobile phase depends on the nature of the compounds being analyzed. The different properties of solvents define whether they are suitable for use as a mobile phase either under reversed phase or normal phase conditions.

The most common solvents used for HPLC are listed below:-

- N-hexane
- Methylene chloride
- Chloroform
- Methyl-t-butyl ether
- Tetrahydrofuron (THF)
- Isopropanol (IPA)
- Acetonitrile (ACS)
- Methanol
- Water

A blend of two or more of these solvents is used as the mobile phase in a HPLC analysis. The proportions of the different solvents in the blend act to adjust the polarity of the mobile phase. This is combined with the stationary phase to achieve the separation of mixture. Ideally, the components in the mixture will be separated fully and will elute within a practical time scale.

The most important property of the solvent is its ability to interact with both ifee stationary phase and the analytes in the mixture, resulting in the desired

separation. However, there are other important properties that need to be considered. An ideal solvent will be readily available in high purity, relatively inexpensive, safe to use routinely and compatible with the entire HPLC system including the detector.

The HPLC system:

Instrumentation is required to enable the flow of the mobile phase through the stationary phase and also to convert the separated components in to meaningful information. A typical configuration of a HPLC system is shown below.

Components of HPLC system:

1. **Mobile phase reservoir :-** Stores the mobile phase required for analysis
2. **Degasser :-** Degasses the mobile phase
3. **Pump :-** Solvent delivery system, enables the flow of the mobile phase through the system
4. **Injector :-** Sample delivery system
5. **Column temperature :-** Used to control the temperature of the column
6. **Detector :-** Detects each component in a separated mixture after it has eluted from the column
7. **Data processor :-** Converts the data from the detector in to meaningful results
8. **Waste :-** Collection of the liquid waste

INTRODUCTION TO HIGH PERFORMANCE LC

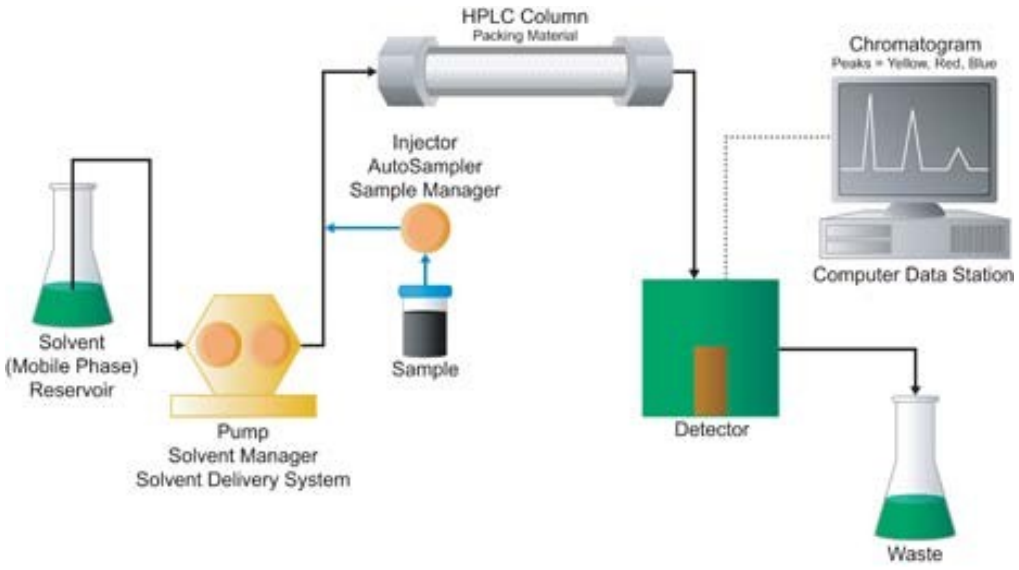


Figure 1: High-Performance Liquid Chromatography [HPLC] System

High performance liquid chromatography is now one of the most powerful tools in analytical chemistry. It has the ability to separate, identify, and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in trace concentrations as low as parts per trillion (ppt) may easily be identified. HPLC can be, and has been, applied to just about any sample, such as pharmaceuticals, food, nutraceuticals, cosmetics, environmental matrices, forensic samples, and industrial chemicals.

A high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi. This was called high pressure liquid chromatography, or HPLC.

ISOCRATIC AND GRADIENT LC SYSTEM OPERATION

Two basic elution modes are used in HPLC. The first is called isocratic elution. In this mode, the mobile phase, either a pure solvent or a mixture, *remains the same throughout the run.*

The second type is called gradient elution, wherein, as its name implies, *the mobile phase composition changes during the separation.* This mode is useful for samples that contain compounds that span a wide range of chromatographic polarity.

HPLC GRADIENT MIXTURES

HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. For low pressure systems this requires great precision in the operation of the miniature mixing General Introduction valves used and low dispersion flows throughout the mixer. For multi-pump high pressure systems it requires a very precise control of the flow rate while making very small changes of the flow rate.

HPLC PUMPS

Because of the small particles used in modern HPLC, modern LC pumps need to operate reliably and precisely at pressures of 10,000 psi or at least 6,000 psi. . For analytical purposes HPLC pumps should have flow rates that range from 0 to 10 ml/min., but for preparative HPLC, flow rates in excess of 100 ml/min may be required.

HPLC SAMPLE VALVES

Sample valves come between the pump and the column it follows that HPLC sample valves must also tolerate pressures up to 10,000 psi. For analytical HPLC, the sample volume should be selectable from sub micro liter to a few micro liters, whereas in preparative HPLC the sample volume may be even greater than 10 ml.

The valves must deliver a very constant sample size but this is usually attained by the use of a constant size sample loop.

HPLC COLUMNS

HPLC columns are packed with very fine particles (usually a few microns in diameter). The very fine particles are required to attain the low dispersion that give the high plate counts expected of modern HPLC. Plate counts in excess of 25,000 plates per column are possible with modern columns, however, these very high efficiencies are very rarely found with real samples because of the dispersion associated with injection valves, detectors, data acquisition systems and the dispersion due to the higher molecular weight of real samples as opposed to the common test samples. The main consideration with HPLC is the much wider variety of solvents and packing materials that can be utilized because of the much lower quantities of both which are required. In particular very expensive optically pure compounds can be used to make Chiral HPLC stationary phases and may even be used as (disposable) HPLC solvents.

MOBILE PHASE RESERVOIR

A reservoir (Solvent Delivery) holds the solvent (called the mobile phase, because it moves). A high-pressure pump solvent manager is used to generate and meter a specified flow rate of mobile phase, typically milliliters per minute.

INJECTOR

An injector (sample manager or auto sampler) is able to introduce (inject) the sample into the continuously flowing mobile phase stream that carries the sample into the HPLC column. The column contains the chromatographic packing material needed to effect the separation. This packing material is called the stationary phase.

HPLC DETECTORS

UV/Vis spectrophotometers, including diode array detectors, are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers, electrochemical detectors, mass spectrometers, light scattering detectors, radioactivity detectors or other special detectors may also be used. Detector consists of a flow-through cell mounted at the end of the column.

A beam of UV radiation passes through the flow cell and into the detector. As compounds elute from the column, they pass through the cell and absorb the radiation, resulting in measurable energy level changes. Fixed (mercury lamp), variable (deuterium or high-pressure xenon lamp), and multi-wavelength detectors are widely available. Modern variable wavelength detectors can be programmed to

change wavelength while an analysis is in progress. Multi-wavelength detectors measure absorbance at two or more wavelengths simultaneously.

In diode array multi- wavelength detectors, continuous radiation is passed through the sample cell, and then resolved into its constituent wavelengths, which are individually detected by the photodiode array. These detectors acquire absorbance data over the entire UV-visible range, thus providing the analyst with chromatograms at multiple, selectable wavelengths, spectra of the eluting peaks and also peak purity.

Differential refractometers detectors measure the difference between the refractive index of the mobile phase alone and that of the mobile phase containing chromatographic compounds as it emerges from the column.

Refractive index detectors are used to detect non-UV absorbing compounds. Fluorimetric detectors are sensitive to compounds that are inherently fluorescent or that can be converted to fluorescent derivatives either by chemical transformation of the compound or by coupling with fluorescent reagents at specific functional groups.

Potentiometric, voltametric, or polarographic electrochemical detectors are useful for the quantitation of species that can be oxidized or reduced at a working electrode. These detectors are selective, sensitive, and reliable, but require conducting mobile phases free of dissolved oxygen and reducible metal ions. Electrochemical detectors with carbon-paste electrodes may be used advantageously to measure nanogram quantities of easily oxidized compounds, notably phenols and catechols.

Many types of detectors can use with HPLC system like UV-Visible or PDA (Photo Diode Array), RI (Refractive Index), Fluorescence, ECD (Electro Chemical Detector), ELSD (Evaporative Light Scattering detector) and many others hyphenated techniques like MS, MS/MS and NMR as well as evaporative IR.

DETECTOR USED IN TLC

UV and spray method:

Of the three techniques, gas, liquid and thin-layer, TLC is the most universal test method as all components are present on the plate and with appropriate detection techniques, all components can be observed. However, it normally is not as accurate or sensitive as HPLC. TLC has a higher analytical variation than HPLC, although one sees the "whole picture" when appropriate detection schemes are selected.

III. REFERENCE STANDARDS

A reference standard is a highly purified compound that is well characterized. Chromatographic methods rely heavily on a reference standard to provide accurate data. Therefore the quality and purity of the reference standard is very important. Two types of reference standards, chemical and nuclidic, exist. With the latter, the radiolabel purity should also be considered as well as the chemical purity.

Samples and analytical data for methods Validation, the two categories of chemical reference standards are as follows: USP/NF reference standard that does not need characterization, and non-compendial standard that should be of the highest purity that can be obtained by reasonable effort and should be thoroughly characterized to assure its identity, strength, quality and purity.

Chromatographic test methods use either external or internal standards for quantitation

A. An external standard method - is used when the standard is analyzed on a separate chromatograrn from the sample. Quantitation is based on a comparison of the peak area/height (HPLC or GC) or spot intensity (TLC) of the sample to that of a reference standard of the analyte of interest.

B. With an internal standard method -- compound of known purity that does not cause interference in the analysis is added to the sample mixture. Quantitation is based on the response ratio of compound of interest to the internal standard vs the response ratio of a similar preparation of the reference standard (HPLC or GC). This technique is rarely used for TLC methods;

The working concentration is the target concentration of the compound of interest as described in the method. Keeping the concentrations of the sample and the standard close to each other for the external standard method improves the accuracy of the method.

VALIDATION

Validation is a key process for effective quality assurance. "Validation" is established documented evidence, which provides a high degree of assurance that a specific process or equipment will consistently produce a product or result meeting its predetermined specification and quality attributes.

DEFINITIONS BY DIFFERENT AGENCIES:

1. **USFDA** defines validation as "establish the documented evidence which provides a high degree of assurance that a specific process will consistently produce a product of predetermined specifications and quantity attributes".

2. **EUGMP** define validation as "action of proving in accordance with the principle of Good manufacturing practice (GMP), that any material, activity or system actually lead to expected result".

3. **AUSTRALIAN GMP** defines validation as "The action of proving that any material, process, activity, procedure, system, equipment or mechanism used in manufacture or control can and will be reliable and achieve the desire and intended result."

OBJECTIVE OF THE VALIDATION

1. The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, quality, and purity they purport or are represented to possess.

2. Assurance of Quantity

3. Government Regulation

IMPORTANCE OF VALIDATION

1. As the quality of product cannot always be assured by routine quality control because of resting of statistically insignificant number of sample, the validation thus should

2. Provide adequacy and reliability of a system or product to meet the predetermined criteria or attributes providing high degree of confidence that the same level of quality is consistently built into each of finished product from batch to batch.

3. Retrospective Validation is useful for trend comparison of results complains to cGMP to cGLP.

4. For taking appropriate action in case of non-compliance.

TYPES OF VALIDATION

The following are frequently required to be validated on a pharmaceutical process:-

1. Equipment, Environment, Materials, Methods, Controls, Process, Personnel's, Facilities and operating procedure. Based on these, the validation program comprises.

2. Equipment Validation

3. Facility Validation including utilities.

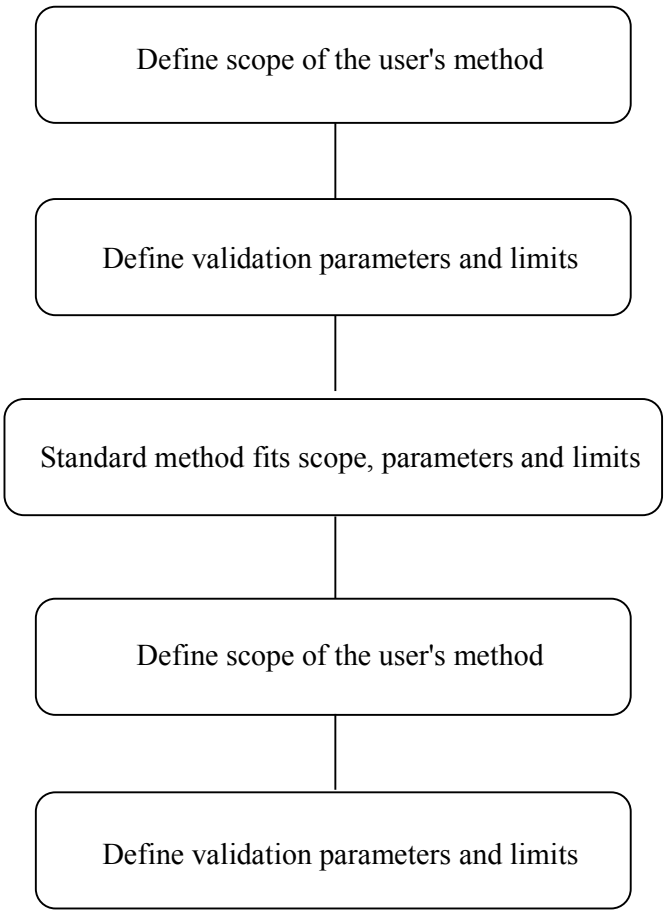
4. Process Validation

5. Cleaning Validation

6. Analytical Method Validation

ANALYTICAL METHOD VALIDATION

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Analytical testing of a pharmaceutical product is necessary to ensure its purity, stability, safety and efficacy. Analytical method validation is an integral part of the quality control system. Although a thorough validation cannot rule out all potential problems, the process of method development and validation should address the most common ones



PARAMETERS USED FOR ASSAY VALIDATION

The validation of the assay procedure was carried out using the following parameters

SPECIFICITY

Definition:

Specificity is the ability to asses unequivocally the analyte in the presence of impurities, degradants, matrix etc (components) which may be expected to be present. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedures.

Determination:

The demonstration of specificity requires that the procedure is unaffected by the presence of impurities or excipients. In practice this can be done by spiking the drug substance or product with appropriate levels of impurities or excipients and demonstrating that the assay result is unaffected by the presence of these extraneous materials.

ICH Requirement:

The ICH documents state that when chromatographic procedures are used, representative chromatograms should be presented to demonstrate the degree of selectivity, and peaks should be appropriately labeled. Peak purity tests (e.g., using diode array or mass spectrometry) may be useful to show that the analyte chromatographic peak is not attributable to more than one component.

ACCURACY

Definition:

The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Determination:

In case of assay of a drug in a formulated product, accuracy may be determined by application of the analytical method to synthetic mixtures of the drug product components to which the known amount of analyte have been added within the range of the method. If it is not possible to obtain all drug product components, it may be acceptable either to add known quantities of the analyte to the drug product or to compare results with those of a second, well characterized method, the accuracy of which has been stated or defined. Accuracy is the measure of how close the experimental value is to the true value. Accuracy studies for drug substance and drug product are recommended to be performed at the 80, 100 and 120% levels of label claim as stated in the Guideline for Submitting Samples and Analytical Data for Methods Validation. For the drug product, this is performed frequently by the addition of known amounts of drug by weight or volume (dissolved in diluent) to the placebo formulation working in the linear range of detection of the analyte. This would be a true recovery for liquid formulations. For formulations such as tablet, suppository, transdermal patch, this could mean evaluating potential interaction of the active drug with the excipients in the diluent. From a practical standpoint, it is difficult to manufacture a single unit with known amount of active drug to evaluate recovery.

This test evaluates the specificity of the method in the presence of the excipients under the chromatographic conditions used for the analysis of the drug product. It will pick up recovery problems that could be encountered during the sample preparation and the chromatographic procedures. However, it does not count the effect of the manufacturing process. At each recommended level studied, replicate samples are evaluated. The RSD of the replicates will provide the analysis variation or how precise the test method is. The mean of the replicates, expressed as % label claim, indicates how accurate the test method is.

ICH Requirement:

The ICH documents recommended that accuracy should be assessed using a minimum of nine determinations over a minimum of three concentration levels, covering the specified range (i.e., three concentrations and three replicates of each concentration.

PRECISION

Definition:

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Precision can be categorized into two types as follows,

System precision:

A system precision is evaluated by measuring the peak response for six replicate injection of the same standard solution prepared as per the proposed method. The RSD is calculating it should not be more than 2%.

Method precision:

A method precision is evaluated by measuring the peak response for six replicate injection of six different weight of sample solution prepared as per the proposed method. The RSD is calculating it should not be more than 2%.

Determination:

The precision of an analytical method is determined by assaying a sufficient number of aliquots of a homogenous sample to be able to calculate statistically valid estimates of standard deviation or relative standard deviation.

ICH Requirement:

The ICH documents recommended that repeatability should be assessed using a minimum of nine determinations covering the specified range for the procedure (i.e., three concentrations and three replicates of each concentration or using a minimum of six determinations at 100% of the test concentration).

LINEARITY

Definition:

The linearity of an analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample

Linearity of an analytical procedure is established, using a minimum of five concentrations. It is established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results are established by appropriate statistical methods, (i.e. by calculation of a regression line by the method of least squares).

ICH Requirement:

ICH recommends that, for the establishment of linearity, a minimum of five concentrations normally be used. It is also recommended that the following minirnum specified ranges should be considered Assay of drug substance (or a finished product) from 80% to 120% of the test concentration.

LIMIT OF DETECTION (LOD)

Definition:

LOD is the lowest concentration of the substance that the method can detect but not necessarily quantify. LOD simply indicates that the sample is below or above a certain level.

Determination:

For non-instrumental methods, the detection limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be reliably detected.

ICH Requirement:

The ICH describes a common approach, which is to compare measured signal from samples with known low concentrations of analyte with those of blank samples.

The minimum concentration at which the analyte can reliably be detected is established. Typically acceptable signal-to-noise ratios are 2:1 or 3:1.

Measurement is based on:

Signal to noise ratio

Visual evaluation (relevant chromatogram acceptable)

The standard deviation of the response and the slope.

$$LOD = \frac{3.3\sigma}{S}$$

Where,

σ - The standard deviation of the response

S - The slope of the calibration curve (of the analyte)

LIMIT OF QUANTITATION (LOQ)

Definition:

LOQ is the lowest concentration of the substance that can be estimated quantitatively with acceptable precision, accuracy and reliability by the proposed method. LOQ is determined by analysis of samples containing decreasing known quantity of the substance and determining the lowest level at which acceptable level of accuracy and precision is attained.

Determination:

For non-instrumental methods, the quantization limit is generally determined by the analysis of samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision

ICH Requirement:

The ICH describes a common approach, which is to compare measured signal from samples with known low concentrations of analyte with those of blank samples. The minimum concentration at which the analyte can reliably be quantified is established. Typically acceptable signal-to-noise ratios are 10:1.

$$LOQ = \frac{10\sigma}{S}$$

Where,

σ - The standard deviation of the response

S - The slope of the calibration curve (of the analyte)

RANGE

Definition:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of anlayte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Determination:

The range of the method is validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.

ROBUSTNESS:

Definition:

The robustness of an analytical procedure is a measure of its capacity to remain unchanged by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Determination:

The robustness of method is determined by performing the assay by deliberately altering parameters (change in flow rate ±10%, change in mobile phase ratio of ±2, change in pH of mobile phase ±0.2, change in wave length detection ±5nm, change in temperature ± 1 to 5°) that the results are not influenced by the changes in the above parameters.

RUGGEDNESS:

Definition:

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different laboratories, different analysts, different instruments, different lots of

reagents, different elapsed assay times, different assay temperatures, different days, etc.

Determination:

The Ruggedness of an analytical method is determined by the analysis of aliquots from homogeneous lots in different laboratories, by different analysts, using operational and environmental condition that may differ but are still within the specified parameters of the assay. The degree of reproducibility of the result is then determined as a function of assay variables. This reproducibility may be compared to the precision of assay under normal condition to obtain a measure of the ruggedness of the analytical method.

SAMPLE SOLUTION STABILITY:

Solution stability of the drug substance or drug product after preparation according to the test method should be evaluated according to the test method. Most laboratories utilize auto samplers with overnight runs and the sample will be in solution for hours in the laboratory environment before the test procedure is completed. This is of concern especially for drugs that can undergo degradation by hydrolysis, photolysis or adhesion to glassware.

SYSTEM SUITABILITY SPECIFICATIONS AND TESTS

The accuracy and precision of HPLC data collected begin with a well behaved chromatographic system. The system suitability specifications and tests are parameters that provide assistance in achieving this purpose. It consists of following factors. .

1. Capacity factor.
2. Precision/injection repeatability
3. Relative retention
4. Resolution
5. Tailing factor
6. Theoretical plate number.

1. CAPACITY FACTOR (k')

K' = (tR - tO) / tf

The capacity factor is a measure of where the peak of interest is located with respect to the void volume, i.e., elution time of the non-retained components.

2. Precision/Injection repeatability (RSD)

Injection precision expressed as RSD (relative standard deviation) indicates the performance of the HPL chromatograph which includes the plumbing, column, and environmental conditions, at the time the samples are analyzed. It should be noted that sample preparation and manufacturing variations are not considered.

3. Relative retention (α)

α = k'1 / k'2

Relative retention is a measure of the relative location of two peaks. This is not an essential parameter as long as the resolution (Rs) is stated.

4. Resolution (Rs)

$$Rs==(t_{R2}-t_{R1}) / (l/2) (t_{w1} + t_{w2})$$

Rs is a measure of how well two peaks are separated. For reliable quantitation, well-separated peaks are essential for quantitation. This is a very useful parameter if potential interference peak(s) may be of concern.

5. Tailing factor (T)

$$T = Wx/2f$$

The accuracy of quantitation decreases with increase in peak tailing because of the difficulties encountered by the integrator in determining where/when the peak ends and hence the calculation of the area under the peak. Integrator variables are preset by the analyst for optimum calculation of the area for the peak of interest. If the integrator is unable to determine exactly when an upslope or down slope occurs, accuracy drops.

6. Theoretical plate number (N)

$$N= 16(tR / tW)^2 = L/H$$

Theoretical plate number is a measure of column efficiency, that is, how many peaks can be located per unit run-time of the chromatogram.

Where,

N - Constant for each peak on a chromatogram with a fixed set of operating conditions.

H - Height equivalent of a theoretical plate.

L - Length of column.

PAPER CHROMATOGRAPHY

Paper chromatography is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a jar containing a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture which starts to travel up the paper with the solvent. This paper is made of cellulose, a polar substance, and the compounds within the mixture travel farther if they are non-polar. More polar substances bond with the cellulose paper more quickly, and therefore do not travel as far.

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is a widely employed laboratory technique and is similar to paper chromatography. However, instead of using a stationary phase of paper, it involves a stationary phase of a thin layer of adsorbent like silica gel, alumina, or cellulose on a flat, inert substrate. For even better resolution and to allow for quantification, high-performance TLC can be used.

TECHNIQUES BY PHYSICAL STATE OF MOBILE PHASE

GAS CHROMATOGRAPHY

Gas chromatography (GC), also sometimes known as Gas-Liquid chromatography, (GLC), is a separation technique in which the mobile phase is a gas. Gas chromatography is always carried out in a column, which is typically "packed" or "capillary".

Gas chromatography (GC) is based on a partition equilibrium of analyte between a solid stationary phase (often a liquid silicone-based material) and a mobile gas (most often Helium).

The stationary phase is adhered to the inside of a small-diameter glass tube (a capillary column) or a solid matrix inside a larger metal tube (a packed column). It is widely used in analytical chemistry; though the high temperatures used in GC make it unsuitable for high molecular weight biopolymers or proteins (heat will denature them), frequently encountered in biochemistry, it is well suited for use in the petrochemical, environmental monitoring, and industrial chemical fields. It is also used extensively in chemistry research.

LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography can be carried out either in a column or a plane. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred as high performance liquid chromatography (HPLC).

In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and

the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more.

AFFINITY CHROMATOGRAPHY

Affinity chromatography is based on selective non-covalent interaction between an analyte and specific molecules. It is often used in biochemistry in the purification of proteins bound to tags. These fusion proteins are labeled with compounds such as His-tags, biotin or antigens, which bind to the stationary phase specifically. After purification, some of these tags are usually removed and the pure protein is obtained. Affinity chromatography often utilizes a biomolecule's affinity for a metal (Zn, Cu, Fe, etc.). Immobilized Metal Affinity Chromatography (IMAC) is useful to separate aforementioned molecules based on the relative affinity for the metal (I.e. Dionex IMAC).

SUPERCRITICAL FLUID CHROMATOGRAPHY

Supercritical fluid chromatography is a separation technique in which the mobile phase is a fluid above and relatively close to its critical temperature and pressure.

Buffer selection

Choice of buffer is typically governed by the desired pH. The typical pH range for reversed-phase on silica-based packing is pH 2 to 8. It is important that the buffer

has a pKa close to the desired pH since buffer controls pH best at their pKa. A rule is to choose a buffer with a pK a value <2 units of the desired mobile phase pH(Table-1).

Table-1: HPLC Buffers, pKa Values and Useful pH Range

Buffer	pKa	Useful pHRange
Ammonium acetate	4.8	3.8-5.8
Ammonium formate	3.8	2.8-4.8
KH2PO4/ phosphoricacid	2.1	1.1-3.1
KH2PO4/K2PO4	7.2	6.2-8.2
Potassium Acetate/ aceticacid	4.8	3.8-5.8
Borate(H3BO3/Na2B4O710H2O)	9.2	8.2-10.2
Ammonium hydroxide/ammonia	9.2	8.2-10.2
Trifluoroacetic acid	<2	1.5-2.5
Potassium formate/formic acid	3.8	2.8-4.8

Buffer concentration

Generally, a buffer concentration of 10-50mM is adequate for small molecules. Generally, no more than 50% organic should be used with a buffer. This will depend on the specific buffer as well as its concentration. Phosphoric acid and its sodium or potassium salts are the most common buffer systems for reversed-phase HPLC.

Selection of detector

Detector is a very important part of HPLC. Selection of detector depends on the chemical nature of analytes, potential interference, limit of detection required, availability and/ or cost of detector. UV-Visible detector is versatile, dual-wavelength absorbance detector for HPLC. This detector offers the high sensitivity

Photodiode Array (PDA) Detector offers advanced optical detection for Waters analytical HPLC, preparative HPLC, orLC/MS system solutions. Refractive Index(RI) Detector offers high sensitivity, stability and reproducibility, which make this detector the ideal solution for analysis of components with limited or no UV absorption. Multi-Wavelength Fluorescence Detector offers high sensitivity and selectivity fluorescence detection for quantitating low concentrations of target compounds.

Column selection

The heart of a HPLC system is the column. Changing a column will have the greatest effect on there solution of analytes during method development. Generally, modern reverse phase HPLC columns are made by packing the column housing with spherical silica gel beads which are coated with the hydrophobic stationary phase. The stationary phase is introduced to the matrix by reacted a chlorosilane with the hydroxyl groups present on the silica gel surface. There are several types of matrices for suppor to the stationary phase, including silica, polymers, and alumina. Silica is the most common matrix for HPLC columns. The nature, shape and particle size of the silica support effects separation. Smaller particle results in a greater number of the oretical plates, or increased separation efficiency. Common stationary phases are C4(butyl),C8(octyl),C18(octadecyl),nitrile(cyanopropyl),andphenyl(phenylpropyl)colu mns. In general, longer alkyl chains, higher phase loading, and higher carbon loads provide greater retention of non-polar analytes. Commonly used reverse phase columns and their uses are listed below. Propyl(C_3), Butyl(C_4), and Pentyl(C_5) columns are useful for ion-pairing chromatography. Examples: include ZorbaxSB-C_3,YMC-PackC4, and LunaC5. These columns are generally less stable to hydrolysis than

columns with longer alkyl chain. Octyl(C_8)columns have wide applicability. This phase is less retentive than the C_{18}phases, but is still quite useful for pharmaceuticals. Example:include(ZorbaxSB-C_8,LunaC_8andYMC-Pack-MOS). Octadecyl (C_{18},ODS) columnsare themost widelyusedandtendtobethemostretentivefornon-polaranalytes. Examples include ZorbaxSB-C_{18},YMC-PackODS and Luna C_{18}. Xterra RP-C_{18} and Zorbax Extend-C_{18} columns have been formulated to tolerate high pH systems (pH>7,normallyuptopH11).

Column temperature

Column temperature control is important for long-term method reproducibility as temperature can affects electivity. A target temperature in the range of 30–40°C is normally sufficient for good reproducibility. Use of elevated temperature can be advantageous for several reasons. The temperature may also affect selectivity patterns because analytes will respond dissimilarly to different temperatures. An increase of 1°C will decrease the k' by1to2%, a both ionic and neutral samples are reported to show significant changes in a with temperature changes. Possible temperature fluctuations during method development and validation, it is recommended that the column be thermostated to control the temperature.

Mobile phase Solvent type

Solvent type (methanol, acetonitrile and tetrahydrofuran) will affect selectivity. The choice between methanol and acetonitrile may be dependent on the solubility of the analyte as well as the buffer used. Tetrahydrofuran is least polar among these three solvent, often responsible for large changes in selectivity and is

also incompatible with the low-wavelength detection required for most pharmaceutical compounds.

Typically a10–50mM solution of an aqueous buffer is used. The most commonly used aqueous phase is H3PO4 in water i.e.phosphatebuffer. The pH of a phosphate buffer is easily adjusted by using mono-, di-, ortribasic phosphate salts. However, when phosphate salts are used the solution should be filtered to remove insoluble particles with 0.22 μm filter paper. Other non-UV active acids and bases may also be used to effect differences in peak shape and retention.

pH of Mobile phase

When the sample is eluted with a mobile phase with 100% organic there is no separation, as the sample is eluted in the void volume. This is because the sample is not retained; but retention is observed when the mobile phase solvent strength is decreased to allow equilibrium competition of the molecules between the bonded phase and the mobile phase. When these parathion is complex, that is, many components are to be separated, and when the solvent strength is decreased and there is still no resolution between two close peaks, another organic solvent of a different polarity or even a mixture of two organics may need to be tried to effect separation. Additionally, mobile phase optimization can be enhanced incombination with bonded phase optimization (i.e., substituting C18/ C8 with cyanoorphenyl). A goal for the band spacing of a solute (K') should be in the range of 4to9and a run time of about15minutes or20minutes at most for most routine product release or stability runs.

Separation Techniques

Isocratic separations: Isocratic, constant eluent composition means equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant; analyte eluent and analyte-stationary-phase inter actions are also constant throughout the whole run. This makes isocratic separations more predictable, although these parathion power (the number of compounds which could be resolved) is not very high. The peak capacity is low; and the longer the component is retained on the column, the wider is the resultant peak.

Gradient separation: Gradient separation significantly increases the separation power of a system mainly because of the dramatic increase of the apparent efficiency (decrease of the peak width). The condition where the tail of a chromatographic zone is always under the influence of a stronger eluent composition leads to the decrease of the peak width. Peak width varies depending on the rate of the eluent composition variation (gradient slope).

Changing Gradient: Gradient elution is employed for complex multicomponent samples since it may not be possible to get all components eluted between k(retention factor) 1and10 using a single solvent strength under isocratic conditions. This leads to the general elution problem where no one set of conditions is effective in eluting all components from a column in a reasonable time period while still at taining resolution no feach component. Employing gradients shall owor steep allows for obtaining differences in the chromatographic selectivity. This would be attributed to the different slopes of the retention versus organic composition for each analyte in the mixture.

Sample preparation for method development

The drug substance being analyzed should be stable in solution (diluent). During initial method development, preparations of the solutions in amber flasks should be performed until it is determined that the active component is stable at room temperature and does not degrade under normal laboratory conditions. The sample solution should be filtered ;the use of a 0.22 or 0.45µm pore-size filter is generally recommended for removal of particulates. Filtration is a preventive maintenance tool for HPLC analysis.

Sample preparation is a critical step of method development that the analyst must investigate. The effectiveness of the syringe filters is largely determined by their ability to remove contaminants/ insoluble components without leaching undesirable artifacts (i.e., extractable) into the filtrate. If any additional peaks are observed in the filtered samples, then the diluents must be filtered to determine if a leachable component is coming from the syringe filter housing/ filter.

Method Optimization

The experimental conditions should be optimized to get desired separations and sensitivity after getting appropriate separations. Stability indicating assay experimental conditions will be achieved through planned/ systemic examination on parameters including pH (if ionic), mobile phase components and ratio, gradient, flow rate, temperature, sample amounts, Injection volume and diluents solvent type.

Method Validation

Validation of analytical procedure is the process by which it is established ,by laboratory studies, that the performance characteristics of the procedure meet the requirements for its intended use. The methods validation process for analytical procedures begins with the planned and systematic collection by the applicant of the validation data to support analytical procedures. All analytical methods that are intended to be used for analyzing any clinical samples will need to be validated. The validation of analytical methods is done as per ICH guidelines.

Validation parameters

The following are typical analytical performance characteristics which may be tested during methods validation:

- Accuracy
- Precision
- Repeatability
- Intermediate precision
- Linearity
- Detection limit
- Quantitation limit
- Specificity
- Range
- Robustness
- System suitability determination
- Forced degradation studies
- Stability studies

Chapter – 2

Literature Review

2. LITERATURE REVIEW

1. Simultaneous determination of ofloxacin and ornidazole in pharmaceutical dosage by reverse phase high performance liquid chromatography

The method was determined for simultaneous determination of ofloxacin and ornidazole from combined dosage form. The separation of drug was achieved on Zorbax Eclipse C18 (250 x 4.6 mm i.d.) with 5 µ particle size. The mobile phase consisted of a mixture of buffer and acetonitrile (65:35 % (v/v)). The buffer was 0.03 M disodium hydrogen phosphate solution adjusted the pH 3.2 with ortho- phosphoric acid. The detection was carried out at wavelength 284 nm. The mixture of buffer of pH 3.2 and acetonitrile (65:35% v/v) was used as a diluent. The method was validated for system suitability, linearity, accuracy, precision, robustness, stability of sample solution. The method has been successfully used to analyze ofloxacin and ornidazole from combined dosage form. **(Rele. Rajan V. et. al., 2015)**

2. Simultaneous determination of ofloxacin and ornidazole in solid dosage form by RP-HPLC and HPTLC techniques

A chromatographic method developed and validated for simultaneous determination of ofl oxacin and ornidazole in solid dosage form. The method was based on reversed phase high performance liquid chromatography, on Intersil C18 column (250 mm, 4.6 i.d.), using acetonitrile:methanol: 0.025M phosphate buffer, pH 3.0 (30:10:60 % v/v/v) as the mobile phase, at a fl ow rate of 1 ml/min at ambient temperature. Quantifi cation was achieved with UV detection at 318 nm over a

concentration range of 2-40 µg/ml for ofl oxacin and 5-100 µg/ml for ornidazole. The mean retention time of ofl oxacin and ornidazole was found to be 4.04 min and 5.83 min, 6.77 min (isomers), respectively. The amount of ofl oxacin and ornidazole estimated as percentage of label claimed was found to be 100.23 and 99.61%, with mean percent recoveries 100.20 and 100.93%, respectively. **(Puranik Manisha, et.al., 2014)**

3. Method development andvalidation for the simultaneous estimation of ofloxacin and ornidazole in tablet dosage form by rp-hplc

A simple reverse phase liquid chromatographic method has been developed and subsequently validated for simultaneous determination of Ofloxacin and Ornidazole in combination. The separation was carried out using a mobile phase consisting of 2mM phosphate buffer and Acetonitrile with pH 3.5 adjusted with ortho phosphoric acid in the ratio of 70: 30%v/v. The column used was Phenomenex C18, (250 mm x 4.6 mm i.d, 5m) with flow rate of 1 ml / min using PDA detection at 293 nm. The described method was linear over a concentration range of 5-50 g/ml and 12.5-125 g/ml for the assay of Ofloxacin and Ornidazole respectively. Gatifloxacin (50g/ml) was used as internal standard. The retention times of Ofloxacin, Ornidazole and Gatifloxacin were found to be 2.1, 2.5 and 5.5min respectively. Results of analysis were validated statistically and by recovery studies. The limit of detection (LOD) and the limit of quantification (LOQ) for Ofloxacin and Ornidazole were found to be 5 and10 µg/ml 10 and 25 µg/ml respectively. **(Dhandapani B. et. al., 2010)**

4. Development and validation of a method for simultaneous estimation of ofloxacin and ornidazole in different dissolution media

A spectrophotometric method was developed for determination of ofloxacin and ornizole in bulk and in pharmaceutical preparations. The method is based on the formulation in buffer pH 6.8 and 7.4 for ofloxacin and ornidazole respectively. The drugs were quantitatively measured at 294 and 287 nm for ofloxacin and ornidazole respectively. **(Patel Dasharath M. et. al., 2012)**

Recovery Studies of drug mixture-

Media	Physical mixtures		Concentration of ofloxacin (µg/ml)	% Recovery	Concentration of ornidazole (µg/ml)	% Recovery
	Ofloxacin (µg/ml)	Ornidazole (µg/ml)				
0.1 N HCl	4	10	4.1	102.50	9.013	90.13
	5	15	4.68	93.60	14.70	98
	6	20	6.33	105.50	19.78	98.90
Phosphate buffer pH 6.8	4	10	4.36	109	10.5728	105.72
	5	15	5.34	106.80	14.54	96.93
	6	20	6.36	106	19.27	96.35
Phosphate buffer pH 7.4	3	10	2.75	91.66	9.13	91.30
	4	15	3.71	92.75	13.62	90.80
	5	20	4.78	95.60	19.37	96.85

5. Development of uv-spectrophotometric method for the quantitative estimation of ofloxacin and ornidazole in combined liquid oral dosage form by simultaneous equation method

A simple, sensitive and accurate UV-Spectrophotometric method for the quantitative estimation of Ofloxacin and Ornidazole in bulk and combined liquid oral dosage form has been developed and validated. Methanol was used as a solvent for estimation of ofloxacin and ornidazole in combined liquid dosage form. Both the standard solutions were scanned over the range of 400-200 nm in spectrum mode of spectrophotometer at medium scanning speed

using U.V spectrophotometer. The maximum absorbance for Ofloxacin and Ornidazole was found at 295.6 nm and 310.8 nm respectively. Ofloxacin and Ornidazole were found to be in the range of 99.58 –100.69 % and 99.86 – 101 %, respectively. The results of analysis were validated statistically. **(Gandhi V.M. et. al., 2013)**

6. Simultaneous Estimation of Ciprofloxacin Hydrochloride, Ofloxacin, Tinidazole and Ornidazole by Reverse Phase – High Performance Liquid Chromatography

An assay has been developed for simultaneous estimation of Ofloxacin and Ornidazole in tablet formulations. The separation was achieved by using C-18 column (Phenomenax, 250 x 4.6mm i.d.) in mobile phase Acetonitrile: Water: Tri ethylamine (25:75). The pH of mobile phase was adjusted to 6.0 ± 0.1 with 50% ortho phosphoric acid. The flow rate was 1.0 mL.min-1 and the separated drugs were detected using UV detector at the wavelength of 300 nm. The retention time of Ofloxacin and Ornidazole was noted to be 3.5 and 5.8 min, respectively. The method was validated as per ICH guidelines. The proposed method was found to be accurate, reproducible, and consistent. It was successfully applied for the analysis of these drugs in marketed formulations. **(Singh Ranjit et. al., 2009)**

7. Development & validation of RP-UPLC method for simultaneous estimation of ofloxacin and ornidazole in their combine dosage form including stress study

A RP-UPLC method was developed for the analysis of Ofloxacin and Ornidazole in combined infusion form. The sample was analyzed by RP-UPLC instrument using reverse phase C18 column (Purospher Star 100×2.1 mm, Merck

Specialities) as stationary phase and Phosphate Buffer: Acetonitrile (70:30 v/v) as a mobile phase [where PH of of the buffer was adjusted to 2.5 by using Tri ethyl amine (1ml / lit buffer) and ortho-phosphoric acid] at a flow rate of 0. 4 ml/min. TUV detector was used for the detection at 294 nm. The retention time for Ofloxacin and Ornidazole was found to be 0.648 and 1.158 minute respectively. **(Manan Sevak et. al., 2010)**

8. **Simultaneous Determination and Validation of Ofloxacin and Ornidazole in Combined Dosage Pharmaceutical Formulation**

The method was carried out on a Xterra TM ® RP18, 5 µm (4.6 X250mm) column with a mobile phase consisting of acetonitrile : mixed phosohate buffer [0.21012% (w/v) KH2PO4 and 0.10852% (w/v) K2HPO4, in HPLC grade water and pH is adjusted to 3.0 with 10% potassium hydroxide solution.] in the ratio 40 : 60. The flow rate was 1.0 ml/min and the effluent was monitored for ofloxacin at 294 nm and for ornidazole at 305 nm (Waters 2487 dual absorbance detector).The retention time of ofloxacin and ornidazole was 2.84 min and 4.39 min respectively. Percentage recoveries for Ofloxacin and Ornidazole were 99.54% to 97.60% and 99.77% to 98.47% respectively. The validation of the proposed method was also carried out in terms of linearity, accuracy, precision,symmetry factor, plate count, regression, and recovery. **(Ghosh Soumya Jyoti et. al., 2009)**

9. Development And Validation Of UV Spctrophotometric method for determination of Ofloxacin and Ornidazole in combined dosage form using smultanious equation method

A simple, precise UV spectrophotometric method has been developed for determination of ofloxacin and ornidazole in its tablet dosage form by using distilled water as a solvent. The absorption maximum (λ max) was observed at 287 nm and 318 nm respectively. The method obeys beer lamberts linearity in the range of 2-12 ug/ml for ofloxacin and 5- 30 ug/ml for ornidazole with a value of correlation coefficient (R 2) 0.999 for both the drugs at their respective wavelength. Assay was performed on two different brands of marketed formulation. Precision studies were carried out as per ICH guidelines Q2 R1and were found to be within the limits NMT 2 % concluding that the method is precise. The proposed method can be used for routine purity testing in bulk and combined dosage forThe UV study was carried out to determine OFX and ORN by simultaneous equation method in combined dosage form. The objective of this method was to develop a simple precise and reproducible method to analyse both the drugs for routine use. The method was developed using distilled water as a solvent. It involved formation of simultaneous equations using 287 nm and 318 nm as the wavelengths. (Absorption maxima of both the drugs).The absorptivity values (A 1% 1cm) were calculated at both the wavelengths (287 nm and 318 nm). An attempt was made to develop a simple, accurate, precise and economical simultaneous equation method for quantitative determination of OFX and ORN in pharmaceutical tablet dosage form. The optimized method was validated according to ICH guidelines Q2 R1. (**Farrel Lisa Gauncar and Sachi S. Kudchadkar, 2009)**

10. RP- HPLC Method for Simultaneous Estimation of Ofloxacin and Ornidazole from Bulk and Tablets

A simple, selective, rapid, precise and economical reverse phase high-pressure liquid chromatographic method has been developed for the simultaneous estimation of Ofloxacin and Ornidazole from pharmaceutical formulation. The method was carried out on a Kromasil C18(5mm, 25 cm X 4.6 mm,i.d.) column, with a mobile phase consisting acetonitrile: phosphate buffer (pH 2.4) in the ratio 80: 20% V/V at a flow rate of 1.0 ml/min. Detection was carried out at 294 nm. The retention time of Ofloxacin and Ornidazole were 2.773 and 5.448min respectively. The developed method was validated in terms of accuracy, precision, linearity, Limit of detection, Limit of quantitation. The proposed method can be used for estimation of these drugs in combined dosage form for routine analysis. The assay was validated according to ICH guideline. The proposed method was found to be simple and linear in the concentration range of 20 to 100 mg/ml for Ofloxacin and 25 to 125mg/ml for Ornidazole respectively. The method was found to be accurate and precise as indicated by recovery studies and % RSD not more than 1.5. Moreover LOD and LOQ Ofloxacin were found to be 0.002 mg/ml and 0.0061 mg/ml, respectively and for Ornidazole were 0.091mg/ml and 0.2768 mg/ml, respectively. Thus the method is specific and sensitive. (**RS Jadhav, et.al., 2009)**

11. Method Development and Validation for Simultaneous Estimation of Ofloxacin and Ornidazole In Bulk and Pharmaceutical Dosage forms

A simple, rapid, sensitive reverse-phase high-performance liquid chromatography method was developed and validated for simultaneous estimation of ofloxacin and ornidazole, at single wavelength of 343nm.chromotographic separation

was performed on an enable aligent zorabax(thermo) column(250nmx4.6mm ID particle size 5 um) and a mobile phase consisting of acetonitrile and buffer (600:4300v/v) at a flow rate of 1.0ml/min. the calibration curve was linear (r2 ≥0.0999) over the concentration range. 400-1200µg/mL of ofloxacin and 1000-3000µg/mL of ornidazole. the limit of detection 0.00246µg/ml for ofloxacin 0.00508µg/ml for ornidazole and no interference was found by the excipients in the synthetic mixture. The proposed methods were validated for international conference on harmonization guideline for linearity, accuracy, precision, and robustness for estimation of ofloxacin and ornidazole in bulk and synthetic mixture, and The results were found to be satisfactory. A new high performance liquid chromatographic method has been developed and validated for the simultaneous determination of ofloxacin and ornidazole in pure and tablet formulations. It was observed from the validation results that the developed RP-HPLC method is more sensitive, accurate, precise, repeatable with wide range of linear range as compared to the reported method4 .The run time of the present assay is relatively short i.e. less than 10 min, which enable rapid quantitation of samples in the routine analysis of tablet formulation.. Basing on the above aspects it is concluded that the developed RP-HPLC method can be used for the simultaneous determination of ofloxacin and ornidazole in tablet formulations. **(Challa Sudheer, et.al., 2016)**

12. Spectrophotometric Method for Simultaneous Estimation of Atorvastatin and Amlodipine in Tablet Dosage Form

Atorvastatin is an anti hyperlipo proteinemic drug and amlodipine is an antihypertensive drug. A simple, precise, rapid and selective spectrophotometric method has been developed for the simultaneous determination of atorvastatin and

amlodipine in tablet dosage forms. The method involves solving of simultaneous equations based on measurement of absorptivity at two wavelengths 242nm and 364nm. Linearity range for atorvastatin and amlodipine were 1-20µg/ml and 1-50µg/ml respectively. **(Lakshmana Rao et al., 2010)**

13. Simultaneous determination of Amlodipine besylate and Atorvastatin calcium in tabletd osage forms by spectrophotometric methods

A simple, accurate, precise and reproducible UV spectrophotometric method was developed for simultaneous estimation of Amlodipine besylate (AMD) and Atorvastatin calcium (ATR) in tablet dosage form have been developed. First method is simultaneous equation method; in this method 361nm and 246 nm were selected to measure the absorbance of drugs at both wavelengths. The second method is Q-value analysis based on measurement of absorptivity at 238.8 nm (as an iso-absorptive point) and 246 nm. AMD and ATR at their respective maximum wavelength 361 nm and 246 nm and at isoabsorptive point 238.8 nm shows linearity in a concentration range of 0.5-30 µg/mL. Recovery studies range from>99.82% for AMD and >98.09% for ATR in case of simultaneous equation method and >100% for AMD and>98.45% for ATR in case of Q-analysis method confirming the accuracy of the proposed method. The proposed methods are recommended for routine analysis since it is rapid, simple, accurate and also sensitive and specific. (no heating and no organic solvent extraction is required.) (**Talluri Chandrashekar et al., 2010)**

Chapter – 3

Objectives & Aim of the Work

3. OBJECTIVES AND AIM OF THE WORK

The ever increasing number of drugs and their combinations in the market leads to the need for the development of analytical methods for their quality control. The methods have to be such that it takes less time in their development as well as the best accurate and robust results should be obtained. Based on this concept the aim of the research work was to develop novel analytical methods with the aid of statistical approach such as chemo metrics and Quality by Design and to further extend this study to applicability of these methods.

Antibacterials such as Ofloxacin, class of fluroquinolones and Ornidazole, class of nitroimmidazole, are being prescribed and used worldwide for their broad spectrum activity. Ofloxacin by inhibits the super coiling activity of DNA gyrase halting DNA replicant. Most anaerobic pathogens and several Gram positive strains such as Staphylococci and Streptococci are only moderately susceptible to Ofloxacin.

Thus in some clinical situations a combination with other antibacterial agent is needed to broaden antibacterial spectrum. Ornidazole drug belonging to the class of nitroimmidazole has antibacterial spectrum that includes most anaerobes. This is said to involve interference with DNA by a metabolite in which nitro group has been reduced. Single dose Ornidazole is an important alternative agent for the treatment of many conditions, other than nitroimmidazoles.

Most of the pharmaceuticals industries rely upon quantitative chemical analysis to ensure that the raw material used and the final product thus obtained meet certain specifications and to determine how much component is present in the final product.

The number of drug and drug formulations introduced into the market has been increasing at an alarming rate. Standard analytical procedures for these drugs or formulations may not be available and if available may not suit to our actual conditions of use. So it is required to develop newer analytical methods which are accurate, precise, specific, linear, simple and rapid.

The modern methods of choice of assays are High-pressure Liquid Chromatography (HPLC), which requires highly sophisticated equipment, trained personnel, high purity chemicals and proper maintenance.

Literature review reveals that only limited work has been done on simultaneous estimation of these drugs. So in the present work, an effort is being made to develop a newer method of analysis of these drugs in combination with other drugs and evaluated as per ICH guide lines. HPLC is a modern technique which is more reliable, reproducible method for the standardization of both single and combined dosage form. Developed method will then be useful for their simultaneous determination in bulk as well as dosage form.

Our present work aims to develop a new and specific, precise, accurate, linear, simple rapid and cost effective HPLC method for the simultaneous estimation of Ofloxacin and Ornidazole in the liquid dosage form. The scope of our work extend to validate for the developed methods.

The developed method is validated for parameters such as system suitability, precision, accuracy, linearity, ruggedness and robustness and evaluation of analytical method validation report generated for the developed methods as per ICH guidelines.

The aim is **"Simultaneous Estimation and Validation of Ofloxacin And Ornidazole by RP-HPLC Method" and the validation by the developed methods as per ICH guidelines.**

Every drug or drug substance should match certain specifications and to determine how much component is present in the final product.

The number of drug and drug formulations introduced into the market has Standard analytical procedures for these drugs or formulations may not be available and if available may not suit to our actual conditions of use. So it is required to develop newer analytical methods which are simple, easy to available, accurate, precise, specific, linear and rapid.

The modern methods of choice of assays are High-pressure Liquid Chromatography (HPLC), which requires highly sophisticated equipment, trained personnel, high purity chemicals and proper maintenance and gives very good results with accuracy and fastly.

As the aim according to literature survey few analytical methods were reported using Phosphate buffer and acetonitrile and water as solvent. The present aim is to develop new analytical method to estimate the Ofloxacin and Ornidazole in its combined dosage form. As the drug is polar in nature, it was proposed to select RP-HPLC method.

Method development includes the development of initial chromatographic conditions, setting up and optimization of developed chromatographic conditions for the assay of Ofloxacin and Ornidazole.

The developed method is validated for parameters such as system suitability, precision, accuracy, linearity, ruggedness and robustness and evaluation of analytical method validation report generated for the developed methods as per ICH guidelines.

PLAN OF WORK

i) Study of physicochemical properties of drug, (pH, pKa, solubility and molecular weight)

ii) Preparation of drug standard and sample,

iii) Optimization chromatographic conditions like,

   a. Selection of wavelength

   b. Selection of initial separation conditions

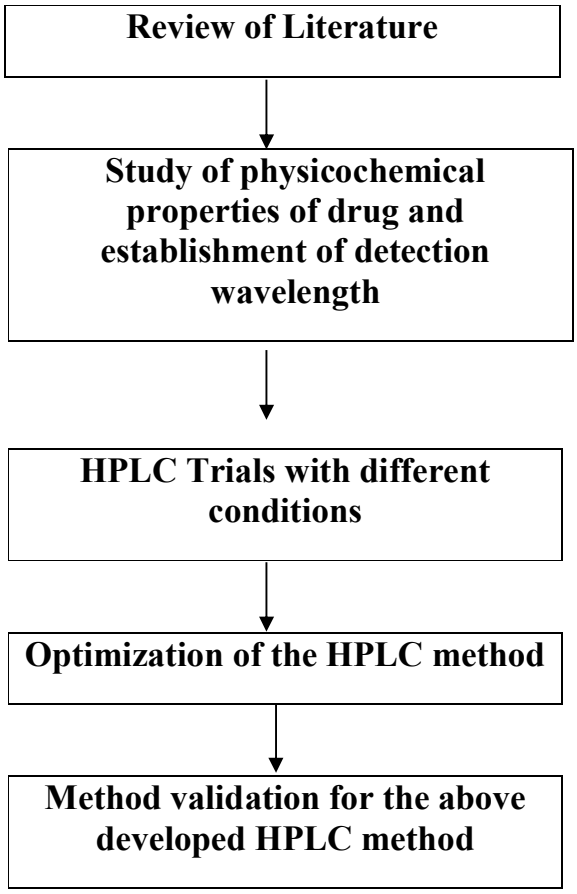
   c. Nature of stationary phase

   d. Nature of mobile phase (pH, solvent strength, solvent ratio and flowrate)

iv) Study of system suitability parameters,

v) Validation of proposed method by RP-HPLC

vi) Applying developed method to marketed formulation.


Review of Literature
Study of physicochemical properties of drug and establishment of detection wavelength
HPLC Trials with different conditions
Optimization of the HPLC method
Method validation for the above developed HPLC method

Chapter – 4

Drug Profile

4. DRUG PROFILE

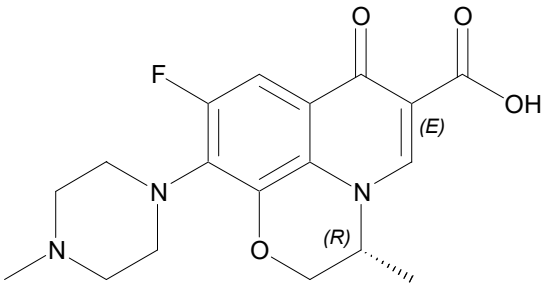
OFLOXACIN

Ofloxacin (OFL) is a fluoroquinolone derivative. Chemically, it is (±)-9-fluoro-2, 3-dihydro-3-methyl10- (4-methyl-1-piperazinyl)-7-oxo-7H-pyrido-[1,2,3-de]- 1,4-benzoxazine -6-carboxylic acid. It is mainly used as antibacterial for the treatment of urinary tract infection and sexually transmitted diseases. Ofloxacin is characterized by a good pharmacokinetic profile. Following oral administration, there is rapid and extensive oral absorption from the gastrointestinal tract achieving peak serum concentration within 1 – 3 h and levels in excess of 100g/ml in the urine and bladder . It is used in the treatment of urinary tract, prostate, skin, urinary and respiratory tract infections. It is also used to treat certain sexually transmitted diseases. Ofloxacin is used as an antibacterial agent in the treatment of infections caused by a wide range of both Gram-positive and Gram-negative bacteria as well as Chlamydia infections.

Ofloxacin is an antibiotic that is used to treat bacterial infections. It belongs to thefluoroquinolone class of antibiotics which includes levofloxacin (Levaquin), ciprofloxacin (Cipro), gatifloxacin (Tequin), norfloxacin (Noroxin), moxifloxacin (Avelox), trovafloxacin (Trovan) and others. Ofloxacin stops the multiplication of bacteria by inhibiting the reproduction and repair of their genetic material (DNA). The FDA approved ofloxacin in December 1990.Ofloxacin is a synthetic, broad spectrum first generation bactericidal fluoroquinolone. **(Dasharath M.Patel, 2012)**

DRUG NAME : OFLOXACINE

STRUCTURE :



CHEMICAL NAME :

(RS)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.05,13]trideca-5(13),6,8,11-tetraene-11-carboxylic acid

MOLECULAR WEIGHT : 361.3675 gm/mole

MELTING POINT : 254 °C

MOLECULAR FORMULAE : $C_{18}H_{20}FN_3O_4$

SOLUBILITY : Soluble in Water

CATEGORY : Antibacterial and anti-infective.

MECHANISM OF ACTION : Ofloxacin acts on DNA gyrase and toposiomerase IV, enzymes which, like huan topoisomerase, prevents the excessive supercoiling of DNA during replication or transcription. By inhibiting their function, the drug thereby inhibits normal cell division.

ABSORPTION : Bioavailability of ofloxacin in the tablet formulation is approx 98%

PROTEIN BINDING : 32%

METABOLISM : Hepatic

REFERENCE

- Hamilton, Richart (2015).
- Susan Blank; Julia Schillinger (2004)
- Knapp JS, Fox KK, Trees DL, Whittington WL (1997).
- Dan M (2004).
- Sato K, Matsuura Y, Inoue M, Une T, Osada Y, Ogawa H, Mitsuhashi S (1982)
- Owens RC, Ambrose PG (July 2005)

DRUG PROFILE

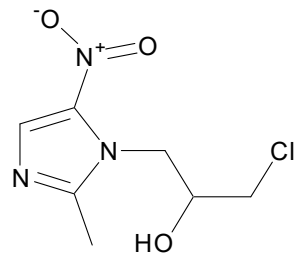
ORNIDAZOLE

Ornidazole is a 5-nitroimidazole derivative and is used in the treatment of susceptible protozoal infections and also in anaerobic bacterial infections. It has been used for amebic liver abscesses, duodenal ulcers, giardiasis, intestinal lambliasis and vaginitis. Ornidazole has recently been used with success in patients with active Crohn's disease. It is more effective against amebiasis than metronidazole, which is the most commonly used nitroimidazole derivative in therapy12- 14. Ornidazole has also been preferred for surgical prophylaxis because of its longer elimination half-life and excellent penetration into lipidic tissues versus other nitroimidazole derivatives. Ornidazole is used in combination with Ofloxacine in the tretment of PLD and in intra-abdominal infection. In this present communication we reports two simple, accurate, and most economical methods for simultaneous estimation of ornidazole and ofloxacin in combained dosage forms. Fixed dose combination of Ornidazole 500 mg and Ofloxacin 200 mg is available in the tablets form in the market. The present work describes a simple, precise and accurate reversed phase HPLC method for the simultaneous estimation of Ornidazole and Ofloxacin in combined dosage form. The method was validated according to procedures and acceptance criteria based on FDA guidelines and recommendations of ICH.

Ornidazole is a antibiotic used to treat some protozoaninfections. It has also been investigated for use in Crohn's diseaseafter bowel resection. **(Gandhi V.M, et.al., 2013)**

DRUG NAME : ORNIDAZOLE

STRUCTURE :



IUPAC NAME : 1-chloro-3-(2-methyl-5-nitro-1H-imidazol-1-yl)propan-2-ol

MOLECULAR WEIGHT : 219.63 gm/mole

MOLECULAR FORMULAE : $C_7H_{10}ClN_3O_3$

CATEGORY : Antibiotic, Antiprotozoal.

MECHANISM OF ACTION : After passive abortion into bacterium cell, the nitro group of ornidazole is reduced into an amine group by redox intermediate intracellular metabolites is believed to be the key component responsible for killing micro-organism.

CONTRAINDICATIONS : Neurological disease blood dyscrasias first trimester of pregnancy hypersensitivity, caution in chronic alcoholics .

ADVERSE EFFECTS : G.I. discomfort, Pain in abdomen, Virtigo Skin rash, Headache.

REFERNECE

- Rutgeerts P, Van Assche G, Vermeire S, et al. (2005)
- Hoffer, Max; Grunberg, Emanuel (1974)
- http://webcache.googleusercontent.com/search?q=cache:OFkC_pjHLZ4J:www.panacea-biotec.com/product-pdf/Ocimix_28-11-2010.pdf

Chapter – 5

Materials & Methods

5. MATERIALS AND METHODS

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) TECHNIQUE

- **MATERIALS USED**

Instruments employed:

1. Digital balance	Wenser
2. pH Meter	Digital pH meter Instrument india
3. Sonicator	Ultrawave, instrument india
4. Membrane filter	Nylon membrane filter (0.45µ)
5. HPLC SHIMADZU-LC 20 AT	
a. Software used	LC Solution
b. Detector	UV-detector
c. Analytical column	Agilent C_{18} (250x4.6mm) 5µ
6. UV SHIMADZU-UV -Visible Spectrophotometer	
a. Instrument Model	UV-1800
b. Instrument Type	UV -1800PC Series
c. Software Name & Version	UV prove 2.21 Version

- **CHEMICALS USED**

Ofloxacin working reference standard	AR grade
Ornidazole working reference Standard	AR grade
Potassium dihydrogen phosphate	AR grade
Acetonitrile	HPLC grade
Ortho phosphoric acid	HPLC grade
Water milli-Q grade	HPLC grade

- **TABLETS BRAND USED: MEDLEY Pharmaceuticals**

Trial – 1:

The trial 1 was performed using mobile phase Acetonitrile and buffer in the ratio 50 : 50 Agilent C_{18} (250 x 4.6 mm) 5µm packing with flow rate 2 ml / min. ofloxacin peak splited.

Trial – 2:

The trial 2 was performed using mobile phase acetonitrile and buffer in the ratio 80 : 20 Agilent C_{18} (250 x 4.6 mm) 5 µ ofloxacin peak splited with flow rate splited (0.1 ml/min.)

Trial – 3:

The trial 3 was performed using mobile phase sodium acetate and buffer in the ratio 55 : 45 agilent C_{18} (250 x 4.6mm) 5 µ packing with flow rate 0.1 ml/min. ofloxacin and onidazole peak splited.

Trial – 4:

The trial 4 was performed using mobile phase ortho phosphoric acid and buffer in the ratio 20 : 80 v/v agilent C_{18} (250 x 4.6mm) 5 µ packing with flow rate 0.1 ml/min ofloxacin and onidazole peak splited.

Trial – 5:

The trial 5 was performed using mobile phase acetonitrile buffer in the ratio 50 : 50 agilent C_{18} (250 x 4.6mm) 5 µ packing with flow rate 0.2 ml/min onidazole peak splited.

Trial – 6:

The trial 6 was performed using mobile phase acetonitrile and buffer in tetra hydro furan in the ratio 35 : 64.9 (pH = 3.5) agilent agilent C_{18} (250 x 4.6mm) 5 µ packing with flow rate 1.0 ml/min ofloxacin peak good separation.

- **METHOD DEVELOPMENT**

Optimization of mobile phase:

Separation of both the drugs was tried using the following combination of mobile phases. The table gives the details of the same

Table No. 1 Method development trails

Serial no.	Mobile phase	Ratio (v/v)	Elution of peak
1.	Buffer : Acetonitrile	50 : 50	Not proper separation
2.	Buffer : Acetonitrile	80: 20	Not proper separation
3.	Buffer : [Sodium Acetate:Methanol : Acetonitrile 5:40 : 55 v/v]	55:45	Separation of peaks
4.	Buffer : [Orthophosphoric acid Acetonitrile : 20 : 80 v/v]	20 : 80	Separation of peaks
5.	Buffer : [Acetonitrile : Orthophosphoric acid 50:50 v/v]	50 : 50	Separation of peaks
6.	**Buffer : [Acetonitrile : Tetrahydrofuron 35:64.9 v/v] Adjust pH with Orthophosphoric acid**	**35-64.9**	**Good Separation**

Out of 6 trials the 6th trial was selected for further studies because when compared to other trails 5th trial was found less in retention time due to the ratio or organic solvent in mobile phase.

Selection of Wavelength

Solution of Ofloxacin and ornidazole were scanned in the UV region and spectrum was recorded (200-400nm). The solvent used was Buffer: [Acetonitrile :

Tetrahydrofuron 35:64.9 v/v] Adjust pH with Orthophosphoric acid in ratio of 35:64.9. It was seen that 300nm both compounds have very good absorbance, which can be used for the estimation of compounds by HPLC.

Selection of Chromatographic Method

Proper selection of the method depends on the nature of the sample (ionic or ionisable or neutral molecules), its molecular weight, pKa value and stability. The drugs selected in the present study are polar and so reversed phase or ion exchange chromatography can be used. The reversed phase HPLC was selected for the initial separation because of its simplicity and suitability.

From the literature survey and with the knowledge of properties of the selected drugs, Aligent C-18 (250x4.6mm) 5µ column was chosen as stationary phase and mobile phase with different compositions such as Potassium dihydrogen phosphate buffer and Acetonitrile was used.

From all the data observed, obtained, available the initial separation conditions were set to work around.

Effect of Ratio of Mobile Phase

Under the chromatographic conditions mentioned above, the different ratios of mobile phase were tried. The chromatograms were observed for each of the trails, out of which Buffer : (Acetonitrile & Sodium acetate & methanol - 55:45 v/v) in the ratio of 50:50 was selected as the separation was achieved in minimum retention time.

Effect of pH of Mobile Phase

Several trials were made using different Buffer solutions of different pH range. The best separation was achieved with Potassium dihydrogen phosphate adjust the pH to 3.5 ± 0.1 with dil. Orthophosphoric acid.

Effect of flow rate on separation

The mobile phase consisting of Buffer : [Acetonitrile : Tetrahydrofuron 35:64:1 v/v] and the chromatograms were recorded at flow rates of 0.5ml to 2ml. The sharp peaks were obtained with 1 ml flow rate.

Effect of column (Stationary phase) on separation

At the chromatographic conditions of mixed solutions, combinations of Ofloxacin and Ornidazole were injected and chromatograms were obtained using C-18 column, so C-18 was preferred for further studies.

Reference Standards

Keeping the all above fixed conditions External standard was used.

Optimized Conditions

The following optimized parameters were used as a final method for the simultaneous estimation of Ofloxacin and Ornidazole.

Instrument	:	SHIMADZU – LC 2010
Column	:	Agilent C18 (250x4.6mm) 5µ
Column Oven Temperature	:	30º C

Wave length	:	300nm
Flow rate	:	1.0ml/min
Injection Volume	:	20µl
Runtime	:	8 minutes
Mode of Operation	:	Reverse phase
		Mobile Phase
Solvent A	:	(Potssium dihydrogen phosphate Buffer
		& Tetra hydro furan 64:01 v/v)
Solvent B	:	(Acetonitrile– 35 v/v)
Solvent ratio	:	64.9 v/v of Solvent A:B

METHOD OF VALIDATION BY – HPLC

❖ SYSTEM SUITABILITY :

Preparation of standard stock solution:

Ofloxacin:

Weigh and transfer accurately 50 mg of Ofloxacin working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

Ornidazole:

Weigh and transfer accurately 62.5mg of Ornidazole working standard into a 50ml clean and dry volumetric flask and make up with 50ml of diluent and sonicate to dissolve.

Preparation of standard solution:

Taken 1.5 ml from above standard stock solution in a 50 ml volumetric flask and diluted with make phase.

Procedure:

System suitability of the method was performed by calculating the parameters namely, resolution and number of theoretical plates on the 10 replicate injection of standard solution into HPLC system and calculated.

❖ **SYSTEM SUITABILITY :**

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations, and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. See pharmacopoeias for additional information. System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based upon the concept that the equipment, electronics, analytical operations and samples to be analysed constitute an integral system that can be evaluated as such. It goes on to mention resolution, column efficiency and peak symmetry as measurements that can be made but it makes no recommendation as to requirements for these parameters.

❖ The tests are based on the concept that the equipment, electronics, analytical operations, and samples analyzed constitute an integral system that can be evaluated as such. Factors that may affect chromatographic behavior include the following: • Composition, ionic strength, temperature, and apparent pH of the mobile phase • Flow rate, column dimensions, column temperature, and pressure • Stationary phase characteristics, including type of chromatographic support (particle-based or monolithic), particle or macropore size, porosity, and specific surface area • Reverse-phase and other surface modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading, and others) RS is a function of the number of theoretical plates, N (also

referred to as efficiency), a, and k. [NOTE—All terms and symbols are defined in Definitions and Interpretation of Chromatograms.] For a given stationary phase and mobile phase, N may be specified to ensure that closely eluting compounds are resolved from each other, to establish the general resolving power of the system, and to ensure that internal standards are resolved from the drug. This is a less reliable means to ensure resolution, as opposed to direct measurement. Column efficiency is, in part, a reflection of peak sharpness, which is important for the detection of trace components. Replicate injections of a standard preparation or other standard solutions are compared to ascertain whether requirements for precision are met. Unless otherwise specified in the individual monograph, data from five replicate injections of the analyte are used to calculate the relative standard deviation (RSD), if the requirement is £2.0%; data from six replicate injections are used if the RSD requirement is >2.0%. .System suitability of method was performed by calculating the chromatographic parameters like column efficiency, resolution, and asymmetric factor on the repetitive injection of standard solution32 . Stability studies 33, 34 The stability studies give an evidence for the variation of quality of drug product under the influence of various environmental factors such as temperature, humidity and light. The international conference of harmonization (ICH) has developed the guideline for stability of new drug substance and product. This guideline gives an idea about the stability data package required for registration. Main aim of carrying out the stability studies was to determine the re-test of the drug substance, establishing the shelf life of the drug products and recommending the proper storage condition of the drug product for long term storage and use. As a whole the stability studies are very much essential because the physical, chemical and microbial variation may

cause a high degree of impact on the efficiency and security of the final drug product.

Table No.2 Result System suitability

Inj no.	Ofloxacin		Ornidazole	
	RT	**Area Response**	**RT**	**Area Response**
1	3.422	1663620	6.077	1688288
2	3.422	1656669	6.077	1688288
3	3.422	1653464	6.077	1688288
4	3.42	1646650	6.07	1688288
5	3.45	1642172	6.12	1689818
6	3.45	1660286	6.12	1693663
7	3.45	1642172	6.124	1685784
8	3.45	1642172	6.124	1682558
9	3.42	1643618	6.07	1685834
10	3.42	1641914	6.077	1688036
Average		1649273.7		1687884.5
STD		7388.3		18955
%RSD		0.447		1.12

Acceptance Criteria:

The %RSD for 10 replicate injections should not more than 2.0%

The system suitability parameters and % RSD for peak areas for 10 replicate injections of standard solution was found to be within limits.

❖ **SPECIFICITY**

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present such as impurities, degradation products and matrix components.

Chromatogram of blank solutions showed no peaks at the retention times of Ofloxacin and Ornidazole. This indicates that the solvents and chemicals used in the formulated do not interfere in estimation of Ofloxacin and ornidazole in the Tablets. An investigation of specificity should be conducted during the validation of identification tests, the determination of impurities, and the assay. The procedures used to demonstrate specificity will depend on the intended objective of the analytical procedure. It is not always possible to demonstrate that an analytical procedure is specific for a particular analyte (complete discrimination). In this case, a combination of two or more analytical procedures is recommended to achieve the necessary level of discrimination. Suitable identification tests should be able to discriminate between compounds of closely related structures which are likely to be present. The discrimination of a procedure may be confirmed by obtaining positive results (perhaps by comparison with a known reference material) from samples containing the analyte, coupled with negative results from samples which do not contain the analyte. In addition, the identification test may be applied to materials structurally similar to or

closely related to the analyte to confirm that a positive response is not obtained. The choice of such potentially interfering materials should be based on sensible scientific judgment with a consideration of the interferences that could occur. For chromatographic procedures, representative chromatograms should be used to demonstrate specificity, and individual components should be appropriately labeled. Similar considerations should be given to other separation techniques. Critical separations in chromatography should be investigated at an appropriate level. For critical separations, specificity can be demonstrated by the resolution of the two components which elute closest to each other. In cases where a nonspecific assay is used, other supporting analytical procedures should be used to demonstrate overall specificity. For example, where a titration is adopted to assay the drug substance for release, the combination of the assay and a suitable test for impurities can be used.

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc. Lack of specificity of an individual analytical procedure may be compensated by other supporting analytical procedure(s). This definition has the following implications: Identification: To ensure the identity of an analyte. Purity Tests: To ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte, i.e., related substances test, heavy metals, residual solvents content, etc. Assay (content or potency): To provide an exact result which allows an accurate statement on the content or potency of the analyte in a sample

Table No.3 Specificity data

Name of Peak	Retention time (Interferences)
Diluent	No time Peak Observed (No interference)
Mobile Phase	No time Peak Observed (No interference)
Ofloxacin	3.4 minutes (No interference)
Ornidazole	6.1 minutes(No interference)

Acceptance Criteria:

Diluent and Mobile Phase should not show any interference at the retention time corresponding to the peak of Ofloxacin and Ornidazole calcium.

Preparation of Standard Stock solution:

Ofloxacin:

Weigh and transfer accurately 50 mg of Ofloxacin working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

Ornidazole:

Weigh and transfer accurately 62.5mg of Ornidazole working standard into a 50ml clean and dry volumetric flask and make up with 50ml of diluent and sonicate to dissolve.

Sample Solution

Weighed and finely powdered not less than 20 tablets. Transferred an accurately weighed portion of the powder of ofloxacin about 100mg and Ornidazole about 250mg in 200ml volumetric flak, added 100ml of diluant phase sonicated for 30 minutes. Make up the volume with diluant. Mixed well and filtered through 0.45µ nylon filter paper discarded first few ml of the filtrate.

Acceptance Criteria

There should not be any interference from blank and diluents solution.

Results for Specificity:

S.NO.	Solutions	Retention time	Peak Purity
1	Diluent	-	-
2	Blank	-	-
3	Ofloxacin and Ornidazole Std.	3.42 and 6.077	Pass
4	Blank	-	-
5	Ofloxacin and Ornidazole Sample	3.422 and 6.07	Pass

❖ **LINEARITY :**

Appropriate aliquots of (0.5, 1, 1.5, 2 & 2.5ml) two drug combination were pipette out from the stock solution into a series of 50ml volumetric flasks. The volume was made up to the mark with Makeup phase. Inject 20µl of each concentration into the HPLC system and chromatographed under the optimized conditions. Evaluation was performed with the UV detector set at 300 nm and the

peak areas were recorded. The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.A linear relationship should be evaluated across the range (see section 3) of the analytical procedure. It may be demonstrated directly on the drug substance (by dilution of a standard stock solution) and/or separate weighings of synthetic mixtures of the drug product components, using the proposed procedure. The latter aspect can be studied during investigation of the range. Linearity should be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may have to be subjected to a mathematical transformation prior to the regression.analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares should be submitted. A plot of the data should be included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. Some analytical procedures, such as immunoassays, do not demonstrate linearity after any transformation. In this case, the analytical response should be described by an appropriate function of the concentration (amount) of an analyte in a sample. For the establishment of linearity, a minimum of five concentrations is recommended. Other approaches should be justified.

Procedure for preparation of standard stock solution

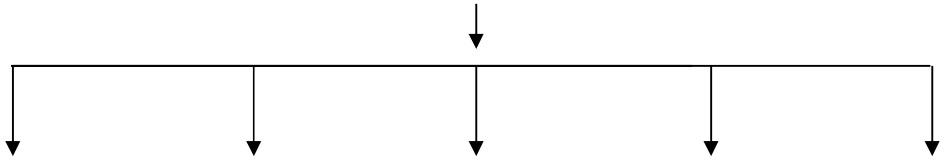
Ofloxacin:

Weigh and transfer accurately 50 mg of Ofloxacin working standard into a 100ml clean and dry volumetric flask and make up with 100ml of diluent and sonicate to dissolve.

Ornidazole:

Weigh and transfer accurately 62.5mg of Ornidazole working standard into a 50ml clean and dry volumetric flask and make up with 50ml of diluent and sonicate to dissolve.

STOCK SOLUTION



	0.5/50 ml M.P.	1/50ml M.P.	1.5/50 ml M.P.	2/50mlM.P.	2.5/50 ml M.P.H_2O
(Oflox)	5 µg	10µg	15 µg	20 µg	25 µg
(Ornda)	12.5 µg	25µg	37.5 µg	50µg	62.5µg

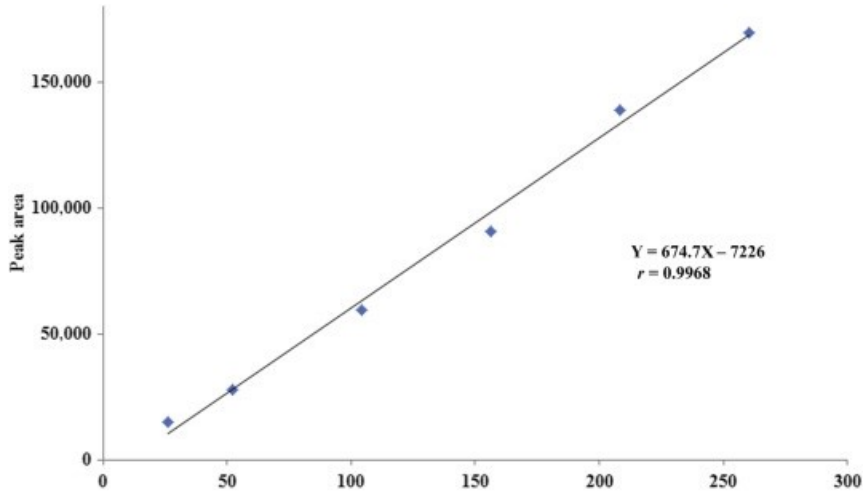


Table No.4 Linearity of Ofloxacin

Conc. (µg/ml)	Area Response	Avg. Area response
5	478739	476859.33
	478306	
	473533	
10	936507	936179.33
	931496	
	940535	
15	1432153	1429202
	1429294	
	1426159	
20	1924976	1929383.67
	1938199	
	1924976	
25	2479720	2479720
	2479720	
	2479720	
	Correlation coefficient	1.012

Acceptance Criteria:

The Correlation Co-efficient should be NLT 0.999 for peak.

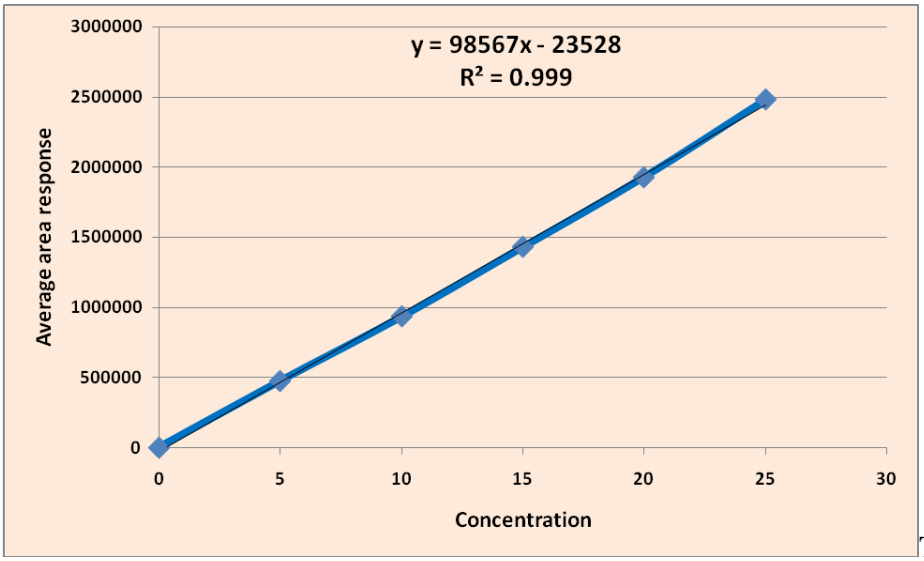
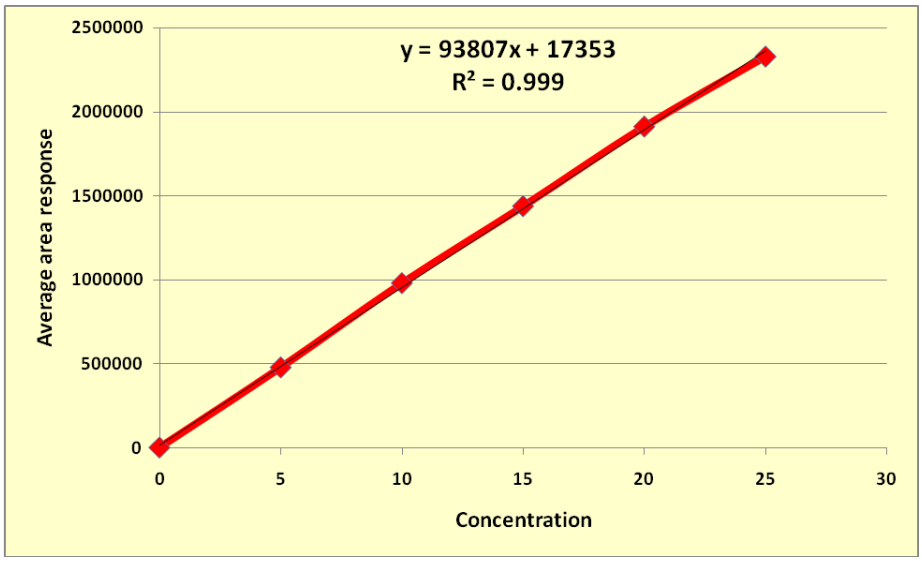


Table No.4 Linearity of Ornidazole

Conc. (µg/ml)	Area Response	Avg. Area response
5	479517	477763.66
	476887	
	476887	
10	982284	979891.33
	973366	
	984042	
15	1442213	1438605
	1438700	
	1434902	
20	1913053	1913053
	1913053	
	1913053	
25	2339249	2330328.67
	2327417	
	2324320	
	Correlation coefficient	1.110

Acceptance Criteria:

The Correlation Co-efficient should be NLT 0.999 for peak.



❖ **ROBUSTNESS:**

❖ The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used. Examples of typical variations are: • Stability of analytical solutions • Extraction time In the case of liquid chromatography, examples of typical variations are: • Influence of variations of pH in a mobile phase • Influence of variations in mobile phase composition • Different columns (different lots and/or suppliers) • Temperature • Flow rate In the case of gas-chromatography, examples of

typical variations are: • Different columns (different lots and/or suppliers) • Temperature • Flow rate

- The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.
- The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters. If measurements are susceptible to variations in analytical conditions, the analytical conditions should be suitably controlled or a precautionary statement should be included in the procedure. One consequence of the evaluation of robustness should be that a series of system suitability parameters (e.g., resolution test) is established to ensure that the validity of the analytical procedure is maintained whenever used. Examples of typical variations are: • stability of analytical solutions, • extraction time In the case of liquid chromatography, examples of typical variations are • influence of variations of pH in a mobile phase, • influence of variations in mobile phase composition, • different columns (different lots and/or suppliers), • temperature, flow rate. In the case of gas-chromatography, examples of typical variations are • different columns (different lots and/or suppliers), • temperature, • flow rate.
- Robustness is the ability of the procedure to provide analytical results of acceptable accuracy and precision under a variety of conditions. The results from separate samples are influenced by changes in the operational or environmental conditions. Robustness should be considered during the

development phase and should show the reliability of an analysis when deliberate variations are made in method parameters.

- Both the ICH and the *USP* guidelines define the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the documentation, providing an indication of the method's or procedure's suitability and reliability during normal use. But while robustness shows up in both guidelines, interestingly enough, it is not in the list of suggested or typical analytical characteristics used to validate a method (again, this apparent discrepancy is changing in recently proposed revisions to *USP*.
- Robustness traditionally has not been considered as a validation parameter in the strictest sense because usually it is investigated during method development, once the method is at least partially optimized. When thought of in this context, evaluation of robustness during development makes sense as parameters that affect the method can be identified easily when manipulated for selectivity or optimization purposes. *Robustness measures the capacity of an analytical method to remain unaffected by small but deliberate variations in method parameters. It also provides some indication of the reliability of an analytical method during normal usage. Parameters that should be investigated are percent organic content in the mobile phase, pH of the mobile phase, buffer concentration, temperature, and injection volume. These parameters may be evaluated one factor at a time or simultaneously as part of a factorial experiment*

- ✓ **Solution Stability:**

Stability of solution is ascertained by determining peak area difference of sample solution at different time intervals with respect to initial results.

Table no. 9 Solution stability data

Time in hours	AMD		ATV	
	% Assay	% Difference	% Assay	% Difference
Initial hour				
12hrs				
24hrs				

$$\% \text{ Difference} = \frac{\text{(Initial value in hour)} - \text{(Difference value in hour)}}{\text{(Initial value in hour)}} \times 100$$

Acceptance Criteria:

- The %Assay should be with in 98% to 102%
- The %Difference should be NMT 2.0

Result:

From the above results, it can be concluded that the standard and sample solution is stable for least 24hrs.

Table No.10 Ruggedness

%RSD from six replicate injection of sample	INSTRUMENTS EMPLOYED	
HPLC Instrument	SHIMADZU (System-1)	WATER ALLIANCE (System-2)
Column	C-18 Aligent (250x4.6mm) 5 µm	C-8 Phenomenex (250x4.6mm) 5 µm
Resolution	11.432	10.288
%RSD Ofloxacin	0.7120	0.2512
%RSD Orndazole	0.5882	0.4661

❖ **PRECISION :**

System precision:

System precision was done by using Ofloxacin and Ornidazole combination of concentration 15 µg/ml each, prepared six times and injected into the HPLC system under the optimized conditions. The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility. Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution. The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.Precision is the degree of agreement among individual results. The complete procedure should be applied repeatedly to separate, identical samples drawn from the same homogeneous batch of material. It should be measured by the scatter of individual results from the mean and expressed as the relative standard deviation (RSD).

Table No.6 System Precision data

Sample no.	Ofloxacin			Ornidazole		
	RT	Area response	Avg.	RT	Area response	Avg.
1.	3.45	1668516	1654190.67	6.12	1699345	1692889
	3.45	1667827		6.12	1696777	
	3.45	1660286		6.124	1693663	
	3.45	1644171		6.124	1691947	
	3.45	1642172		6.12	1689818	
	3.45	1642172		6.12	1685784	
2	3.45	1668747	1667145	6.124	1708789	1701134.33
	3.45	1668747		6.124	1705747	
	3.45	1668747		6.12	1702485	
	3.45	1668516		6.12	1699345	
	3.45	1667827		6.12	1696777	
	3.45	1660286		6.12	1693663	
3	3.45	1640578	1640379.33	6.12	1677269	1670367.33
	3.45	1640578		6.124	1677269	
	3.44	1640578		6.12	1674227	
	3.45	1640578		6.125	1669467	
	3.45	1640578		6.12	1665974	
	3.44	1639386		6.123	1657998	
Average	1653904.67			1688130.33		
STD	27051.67			35526		
%RSD	1.63			2.01		

Acceptance Criteria:

The % RSD should be NMT 2

INTERMEDIATE PRECISION

Intermediate precision (also called within-laboratory or within-device) is a measure of precision under a defined set of conditions: same measurement procedure, same measuring system, same location, and replicate measurements on the same or similar objects over an extended period of time. It may include changes to other conditions such as new calibrations, operators, or reagent lots.

Table No.7 System Intermediate Precision data

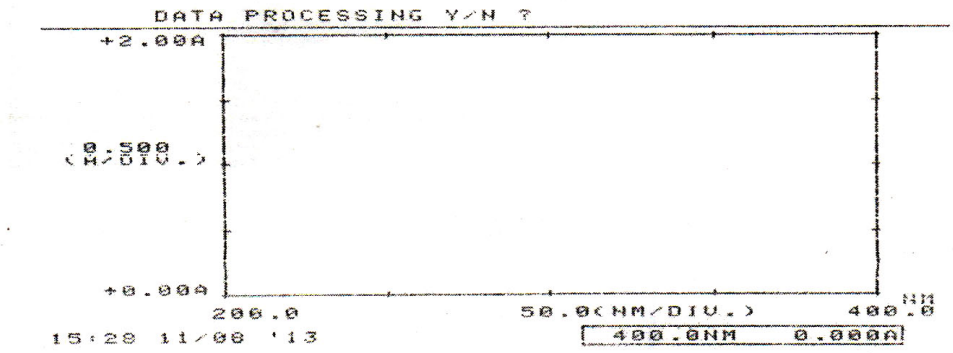
Sample no.	Ofloxacin			Ornidazole		
	RT	**Area response**	**Avg.**	**RT**	**Area response**	**Avg.**
1.	3.42	1653464	1667690	6.077	1685834	1659193
	3.42	1688288		6.077	1676686	
	3.42	1646650		6.077	1669667	
	3.42	1688288		6.077	1654992	
	3.42	1643618		6.077	1640087	
	3.42	1685834		6.077	1627891	
2.	3.42	1641046	1646829	6.077	1688288	1677293
	3.42	1676686		6.077	1688288	
	3.42	1635489		6.077	1676686	
	3.42	1625190		6.077	1685834	
	3.42	1654992		6.077	1669667	
	3.42	1647572		6.077	1654992	
	3.44	1639386		6.123	1657998	
Average	1657260				1668243	

Chapter – 6

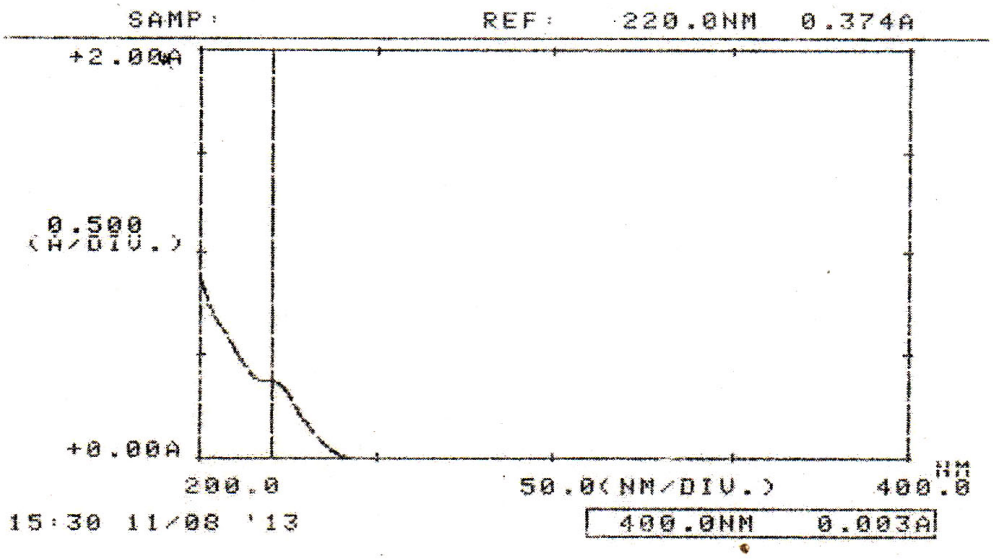
Chromatograms

6. CHROMATOGRAMS

UV Blank



UV Standard



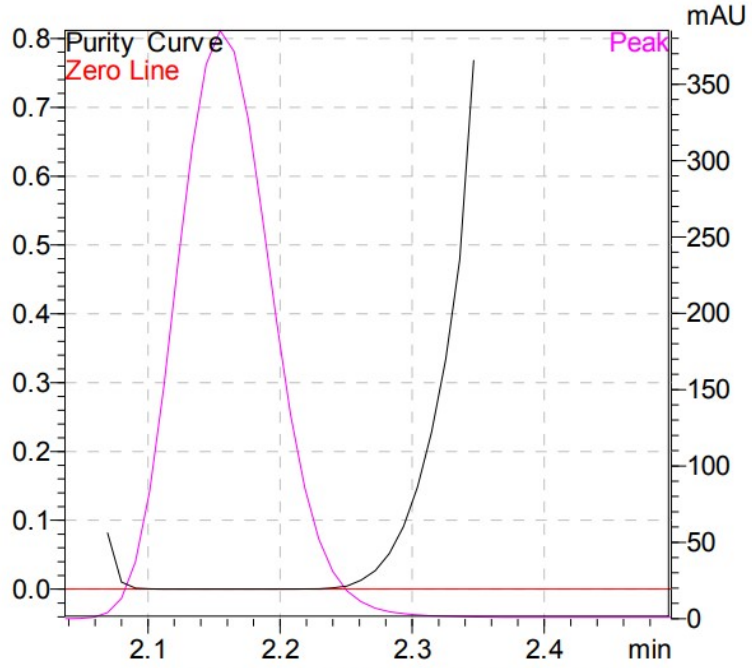


Fig-2 Peak Purity Curve of Ofloxacin

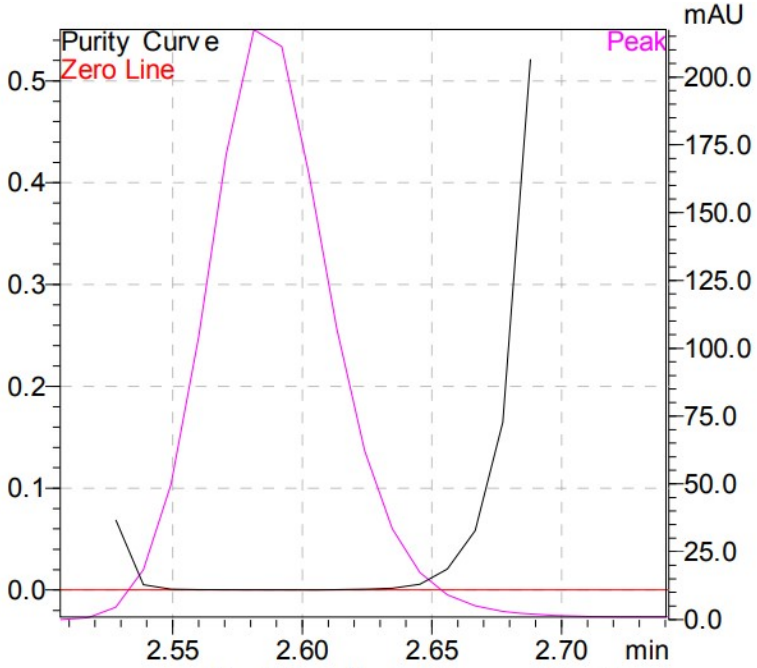
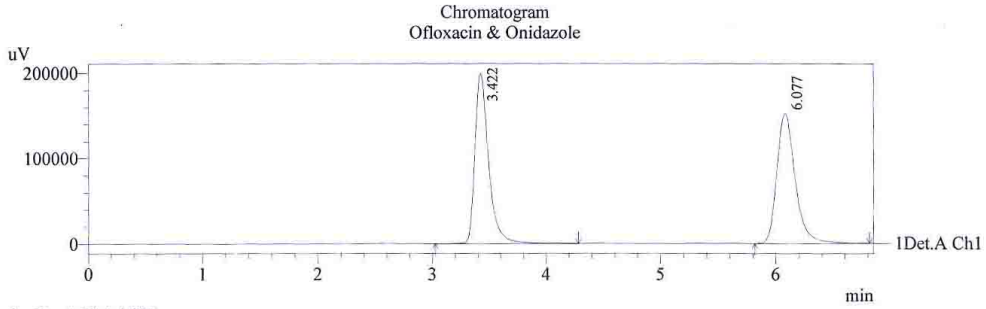


Fig-3 Peak Purity Curve of Ornidazole

ASSAY STANDARD

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	: Std
Tray#	: 1
Vail#	: 13
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm010.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm

PeakTable

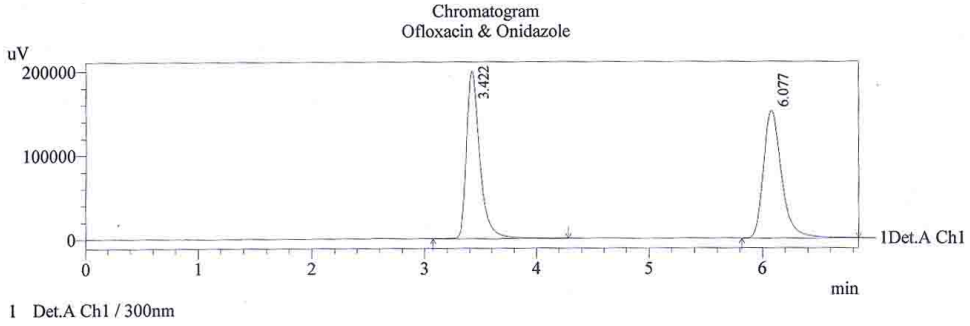
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1666236	199521	49.700	56.710
2	6.077	1686376	152305	50.300	43.290
Total		3352612	351826	100.000	100.000

ASSAY SAMPLE

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	: Sample 01
Tray#	: 1
Vail#	: 14
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm011.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm

PeakTable

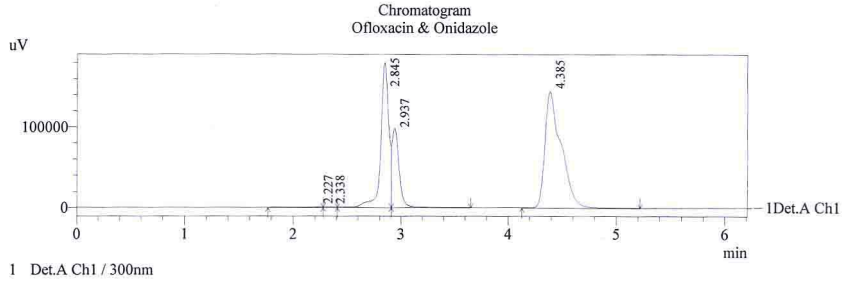
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1665510	199507	49.660	56.706
2	6.077	1688288	152321	50.340	43.294
Total		3353798	351828	100.000	100.000

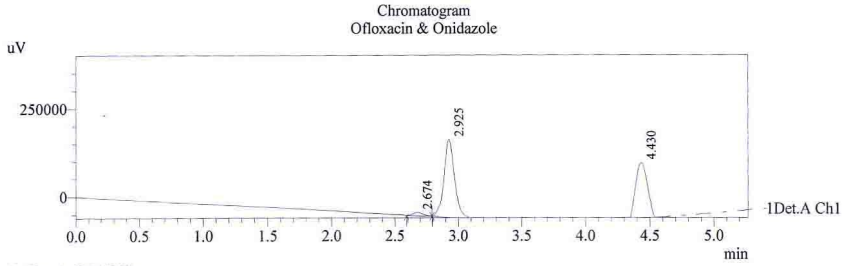
OFLOXACIN + ORNIDAZOLE

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	: Mixed Std
Tray#	: 1
Vail#	: 3
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm013.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.227	4893	338	0.169	0.080
2	2.338	3328	478	0.115	0.113
3	2.845	941347	180404	32.542	42.526
4	2.937	442889	98017	15.311	23.105
5	4.385	1500253	144988	51.863	34.177
Total		2892711	424224	100.000	100.000

PeakTable

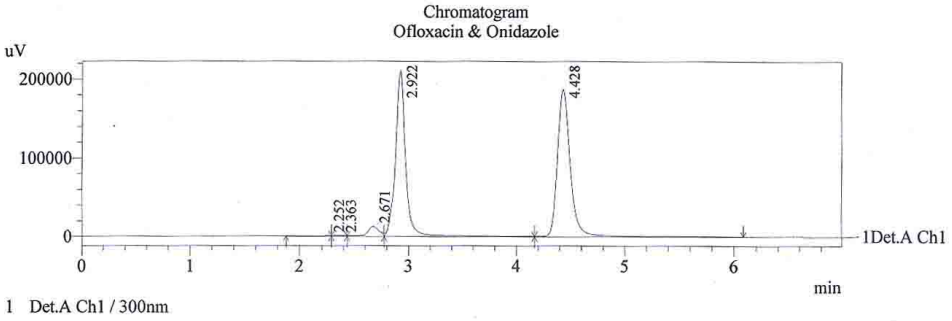
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.674	50721	9429	1.771	2.248
2	2.925	1292704	219813	45.149	52.407
3	4.430	1519777	190195	53.080	45.345
Total		2863201	419437	100.000	100.000

OFLOXACIN + ORNIDAZOLE

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	: Mixed Std
Tray#	: 1
Vail#	: 3
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm017.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm

PeakTable

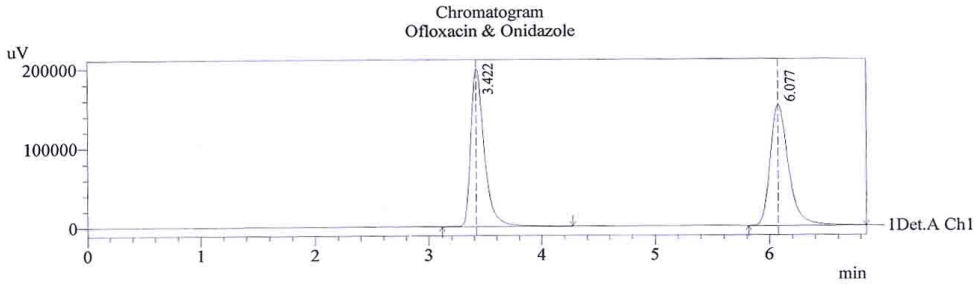
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.252	3715	315	0.124	0.077
2	2.363	3689	550	0.123	0.134
3	2.671	87964	12290	2.933	2.988
4	2.922	1362230	210960	45.417	51.295
5	4.428	1541767	187155	51.403	45.507
Total		2999365	411269	100.000	100.000

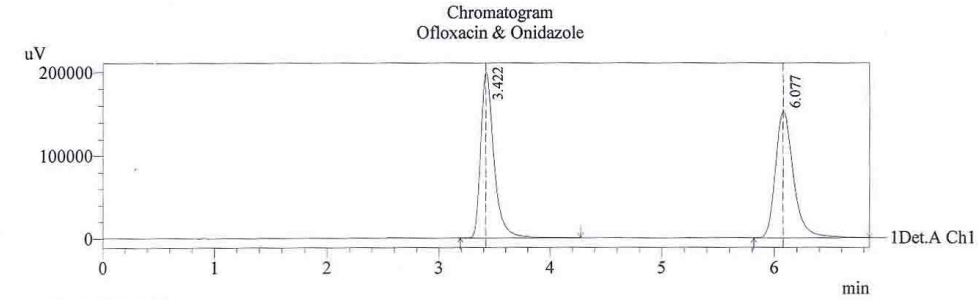
ASSAY SYSTEM SUITABILITY

Sample Information

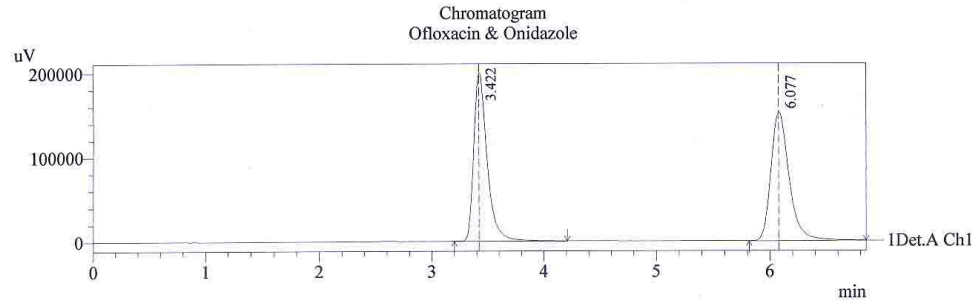
Sample Name	: Ofloxacin & Onidazole
Sample ID	: Assay System Suitability
Tray#	: 1
Vail#	: 15
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm012.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



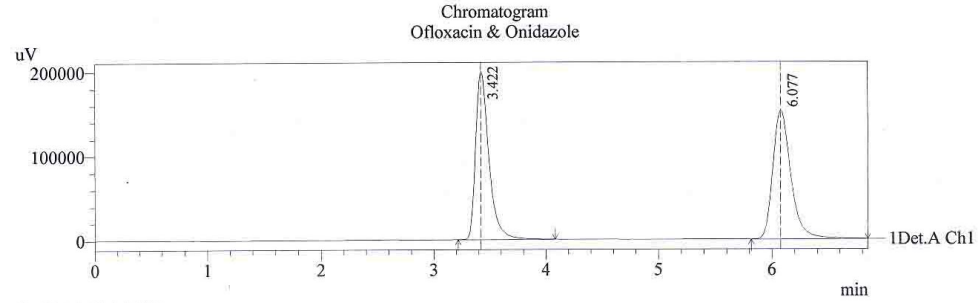
1 Det.A Ch1 / 300nm



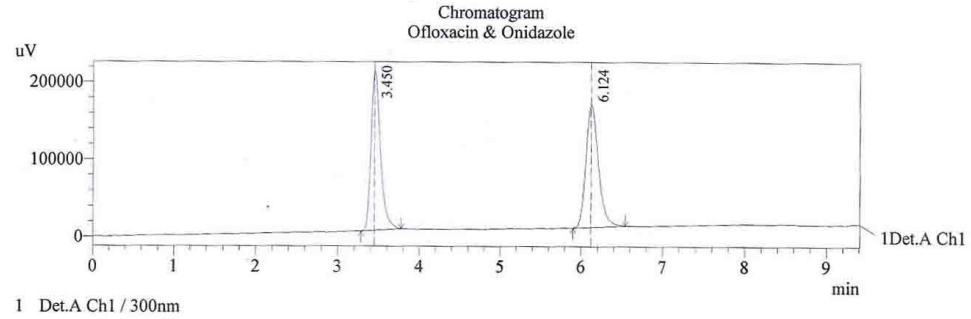
1 Det.A Ch1 / 300nm



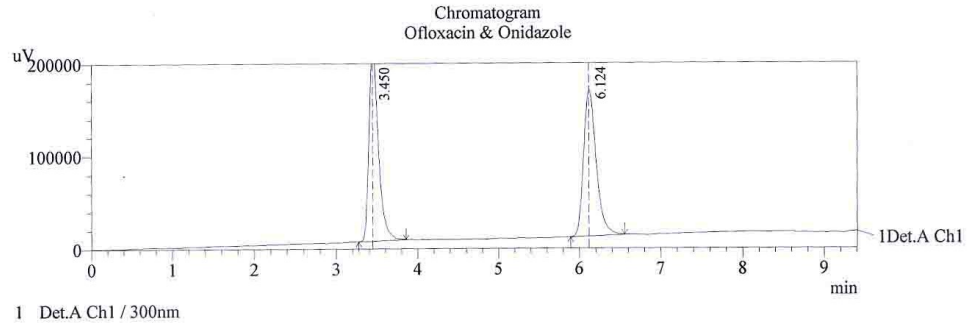
1 Det.A Ch1 / 300nm



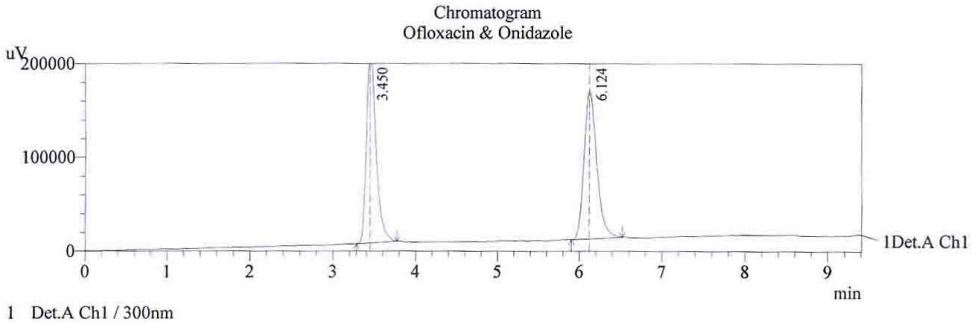
1 Det.A Ch1 / 300nm



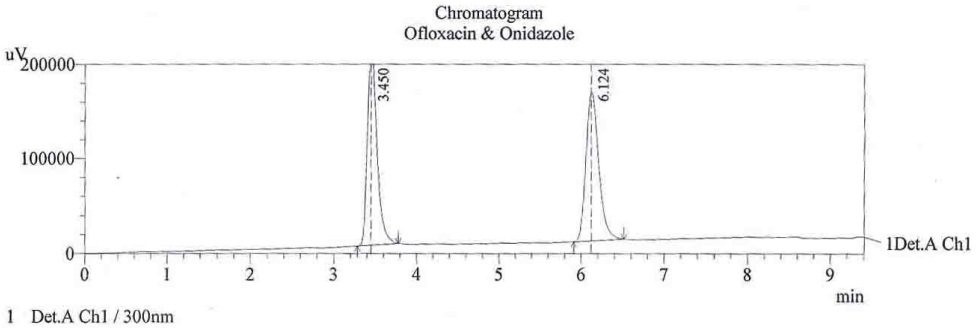
1 Det.A Ch1 / 300nm



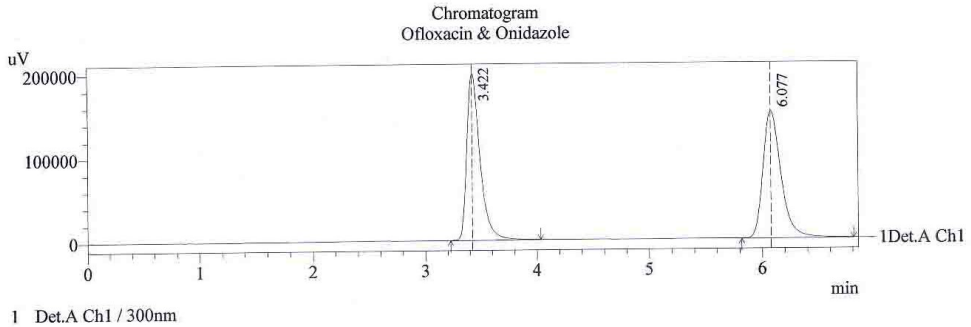
1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1663620	199471	49.632	56.701
2	6.077	1688288	152321	50.368	43.299
Total		3351908	351792	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1656669	199315	49.527	56.682
2	6.077	1688288	152321	50.473	43.318
Total		3344957	351636	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1653464	199279	49.479	56.678
2	6.077	1688288	152321	50.521	43.322
Total		3341753	351600	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1646650	199196	49.376	56.668
2	6.077	1688288	152321	50.624	43.332
Total		3334938	351517	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1642172	205431	49.285	56.668
2	6.124	1689818	157083	50.715	43.332
Total		3331990	362515	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1660286	205796	49.502	56.701
2	6.124	1693663	157156	50.498	43.299
Total		3353949	362952	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1642172	205431	49.345	56.680
2	6.124	1685784	157008	50.655	43.320
Total		3327956	362439	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1642172	205431	49.393	56.692
2	6.124	1682558	156932	50.607	43.308
Total		3324730	362363	100.000	100.000

PeakTable

Detector A Ch1 300nm

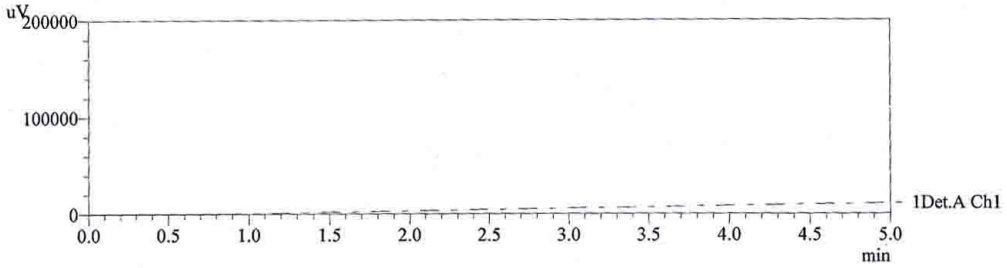
Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1643618	199151	49.366	56.665
2	6.077	1685834	152300	50.634	43.335
Total		3329451	351451	100.000	100.000

SPECIFICITY – BLANK

Sample Information

Sample Name	: Specificity Blank 01
Sample ID	: Specificity Blank 01
Tray#	: 1
Vail#	: 2
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm021.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr

Chromatogram
Onidazole



1 Det.A Ch1 / 300nm

PeakTable

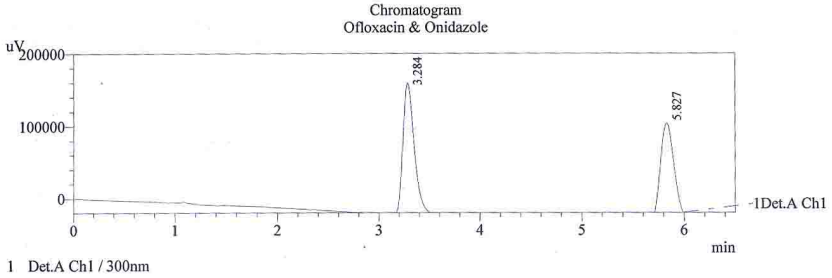
Detector A Ch1 300nm

OFLOXACIN + ORNIDAZOLE

SPECIFICITY

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	: Sample
Tray#	: 1
Vail#	: 21
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm027.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm

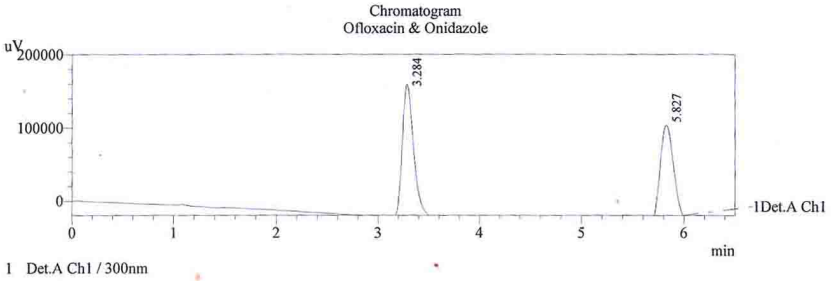
PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.284	1456314	182993	48.890	55.937
2	5.827	1522431	144149	51.110	44.063
Total		2978745	327142	100.000	100.000

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	: Mixed Std
Tray#	: 1
Vail#	: 20
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm026.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm

PeakTable

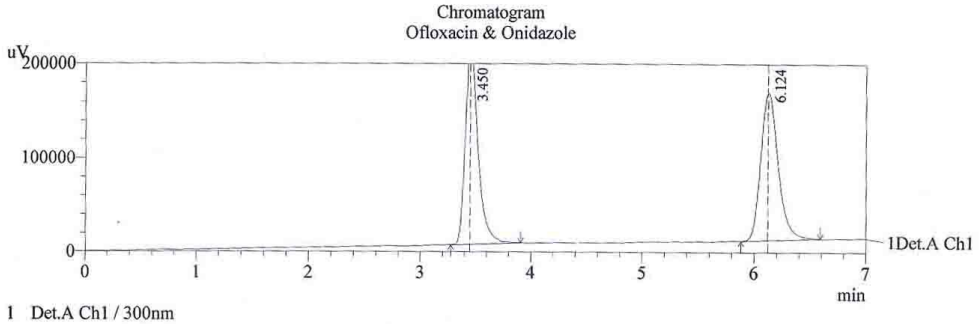
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.284	1456314	182993	48.892	55.937
2	5.827	1522313	144148	51.108	44.063
Total		2978627	327140	100.000	100.000

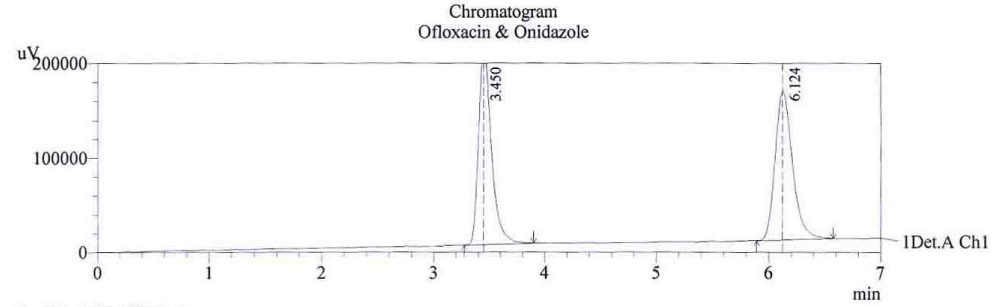
PRECISION SAMPLE

Sample Information

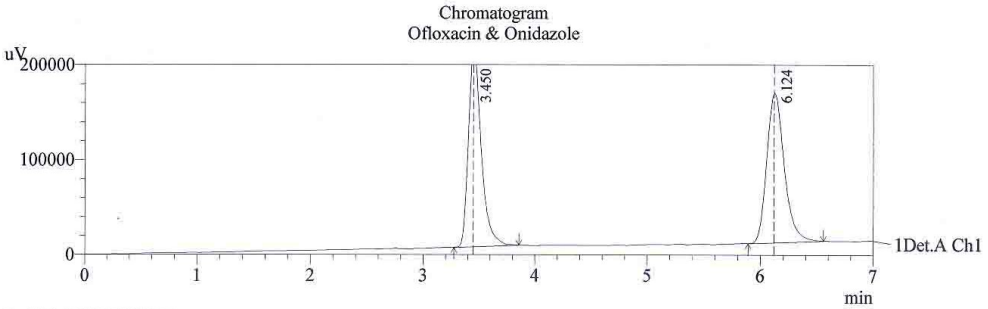
Sample Name	: Ofloxacin & Onidazole
Sample ID	:Method Precision Sample 01
Tray#	: 1
Vail#	: 22
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



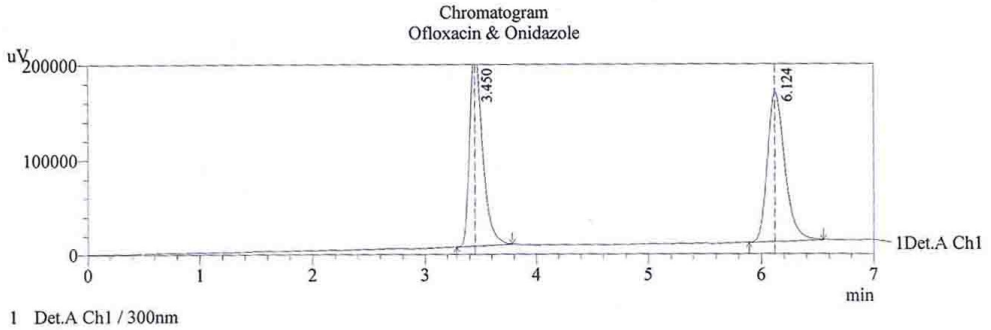
1 Det.A Ch1 / 300nm



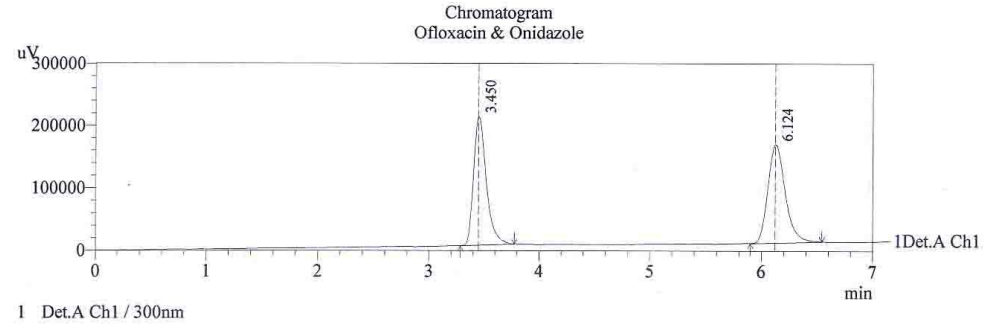
1 Det.A Ch1 / 300nm



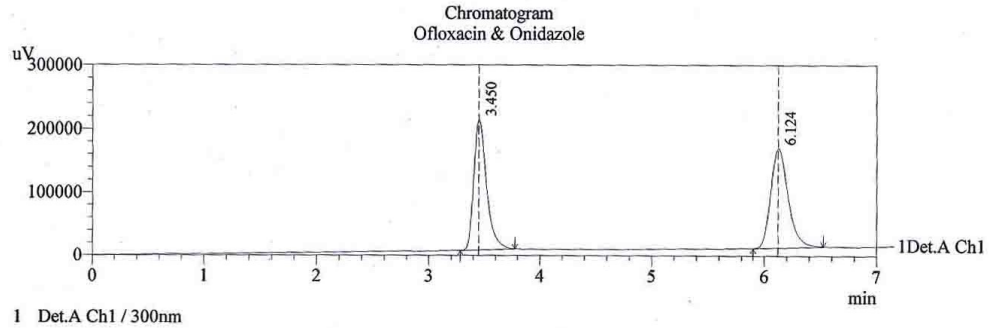
1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1668516	205928	49.542	56.702
2	6.124	1699345	157247	50.458	43.298
Total		3367861	363175	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1667827	205918	49.570	56.707
2	6.124	1696777	157209	50.430	43.293
Total		3364604	363127	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1660286	205796	49.502	56.701
2	6.124	1693663	157156	50.498	43.299
Total		3353949	362952	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1644171	205477	49.284	56.667
2	6.124	1691947	157126	50.716	43.333
Total		3336119	362602	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1642172	205431	49.285	56.668
2	6.124	1689818	157083	50.715	43.332
Total		3331990	362515	100.000	100.000

PeakTable

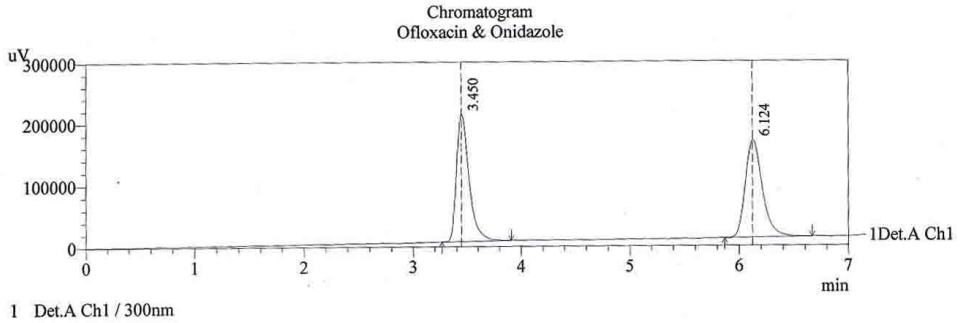
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1642172	205431	49.345	56.680
2	6.124	1685784	157008	50.655	43.320
Total		3327956	362439	100.000	100.000

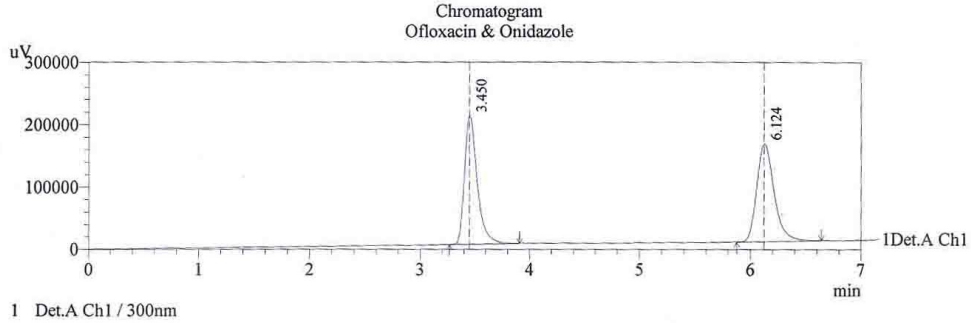
PRECISION SAMPLE

Sample Information

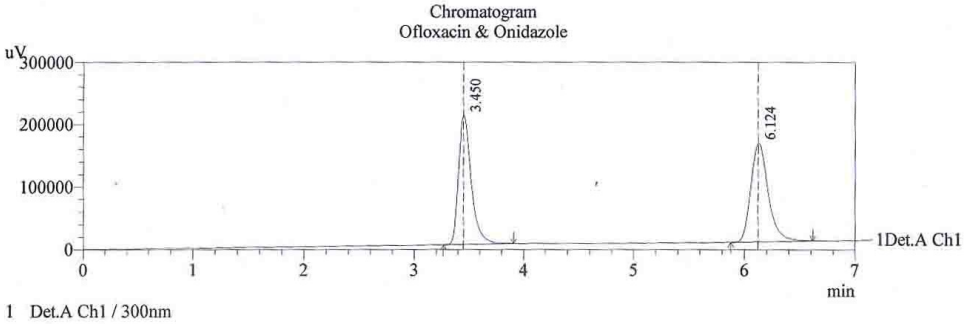
Sample Name	: Ofloxacin & Onidazole
Sample ID	:Method Precision Sample 02
Tray#	: 1
Vail#	: 23
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



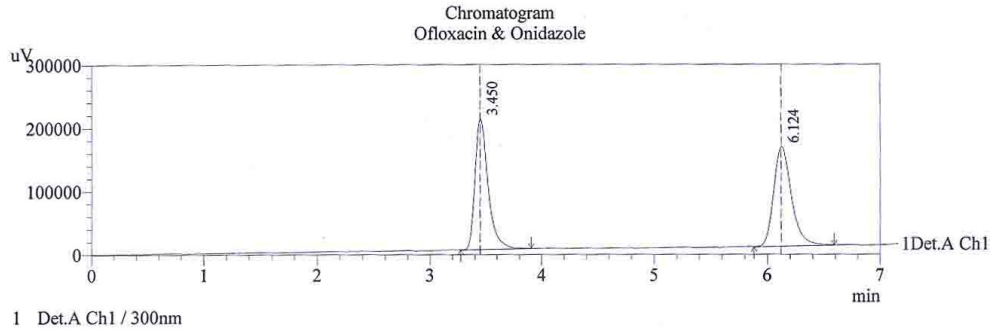
1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1668747	205936	49.350	56.603
2	6.124	1708789	157376	50.534	43.256
3	9.075	3913	513	0.116	0.141
Total		3381449	363825	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1668747	205936	49.394	56.609
2	6.124	1705747	157336	50.490	43.250
3	9.075	3913	513	0.116	0.141
Total		3378407	363785	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1668747	205936	49.500	56.697
2	6.124	1702485	157288	50.500	43.303
Total		3371231	363225	100.000	100.000

PeakTable

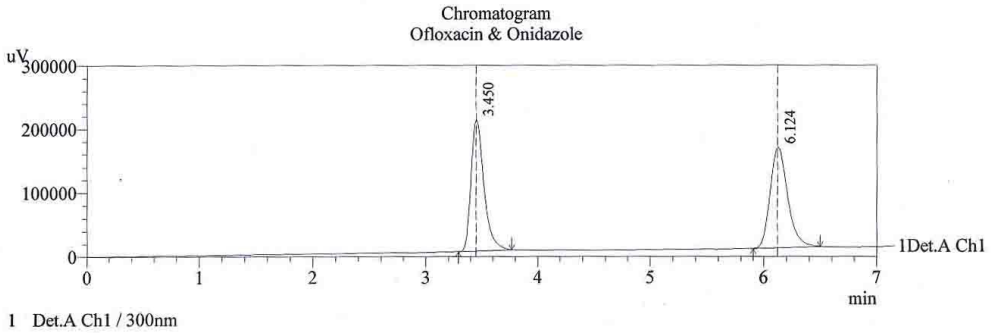
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1668516	205928	49.542	56.702
2	6.124	1699345	157247	50.458	43.298
Total		3367861	363175	100.000	100.000

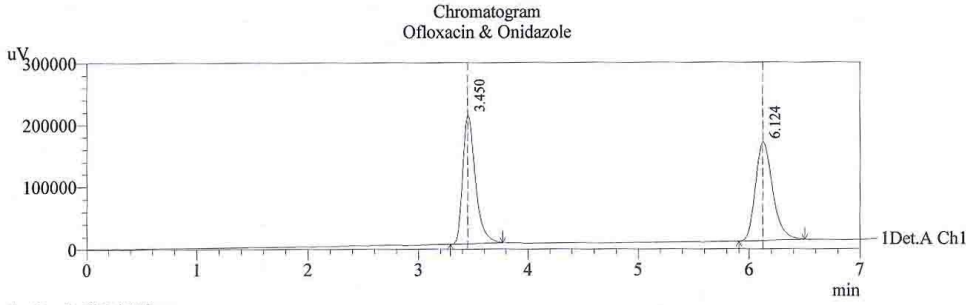
PRECISION SAMPLE

Sample Information

Sample Name	: Ofloxacin & Onidazole
Sample ID	:Method Precision Sample 03
Tray#	: 1
Vail#	: 24
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1640578	205394	49.447	56.704
2	6.124	1677269	156826	50.553	43.296
Total		3317847	362220	100.000	100.000

PeakTable

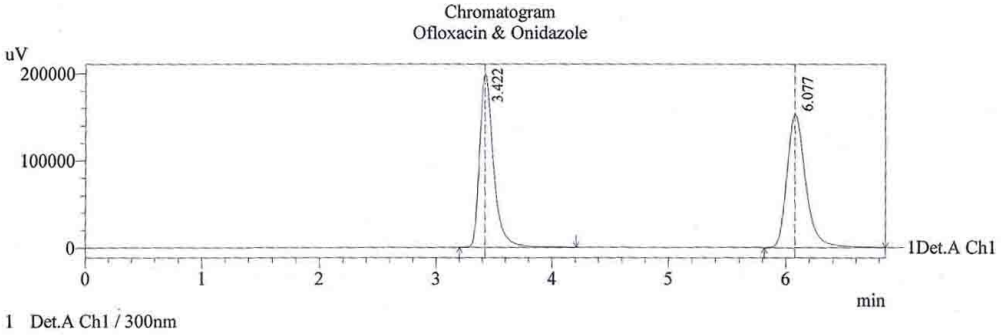
Detector A Ch1 300nm

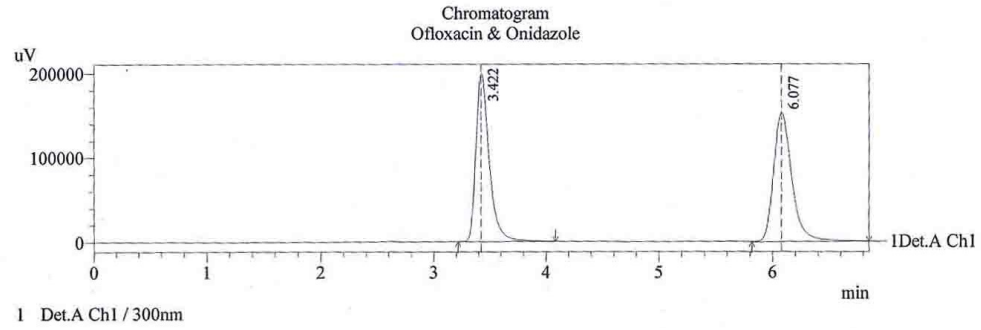
Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.450	1640578	205394	49.447	56.704
2	6.124	1677269	156826	50.553	43.296
Total		3317847	362220	100.000	100.000

INTERMEDIATE PRECISION STANDARD

Sample Information

Sample Name	: Intermediate Precision
Sample ID	: Std
Tray#	: 1
Vail#	: 4
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr





PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1653464	199279	49.479	56.678
2	6.077	1688288	152321	50.521	43.322
Total		3341753	351600	100.000	100.000

PeakTable

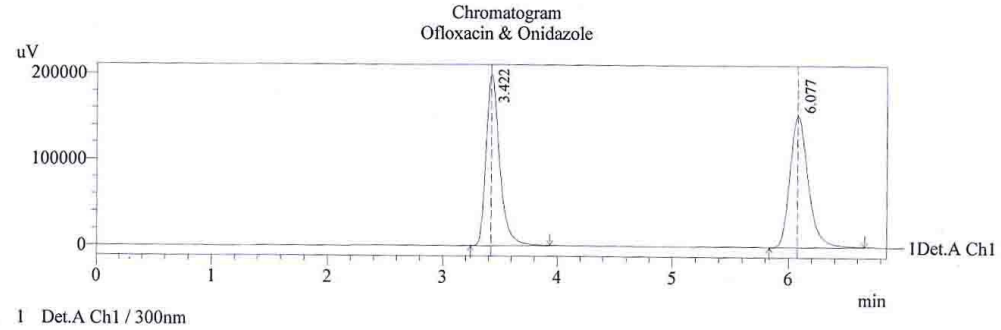
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1646650	199196	49.376	56.668
2	6.077	1688288	152321	50.624	43.332
Total		3334938	351517	100.000	100.000

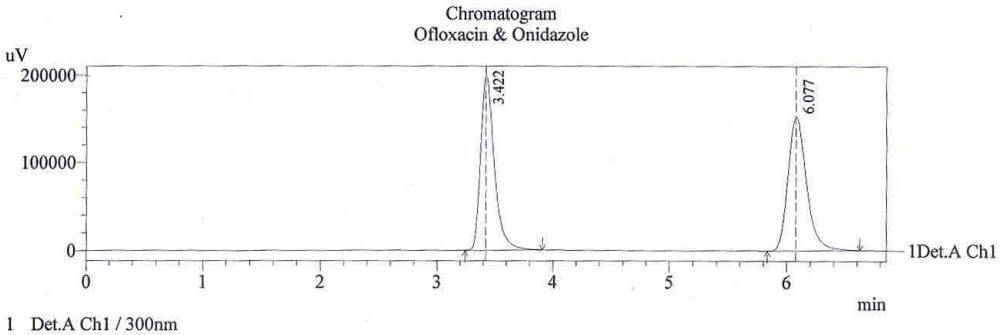
INTERMEDIATE PRECISION SAMPLE

Sample Information

Sample Name	: Intermediate Precision
Sample ID	: Sample Preparation 01
Tray#	: 1
Vail#	: 5
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm02.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1635489	199022	49.427	56.672
2	6.077	1673439	152162	50.573	43.328
Total		3308928	351184	100.000	100.000

PeakTable

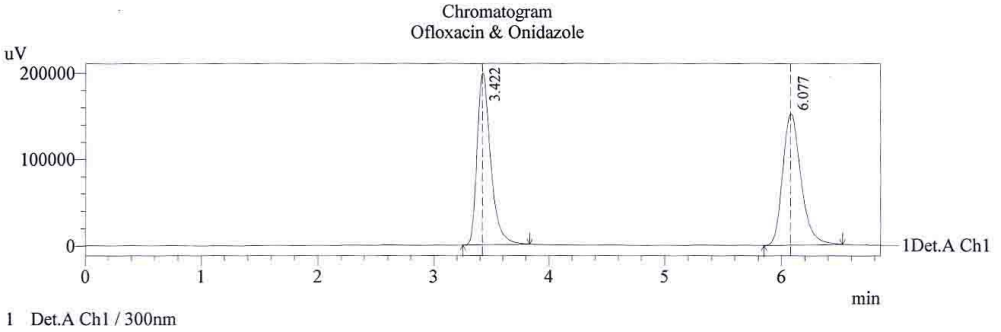
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1633355	198994	49.450	56.676
2	6.077	1669667	152114	50.550	43.324
Total		3303022	351109	100.000	100.000

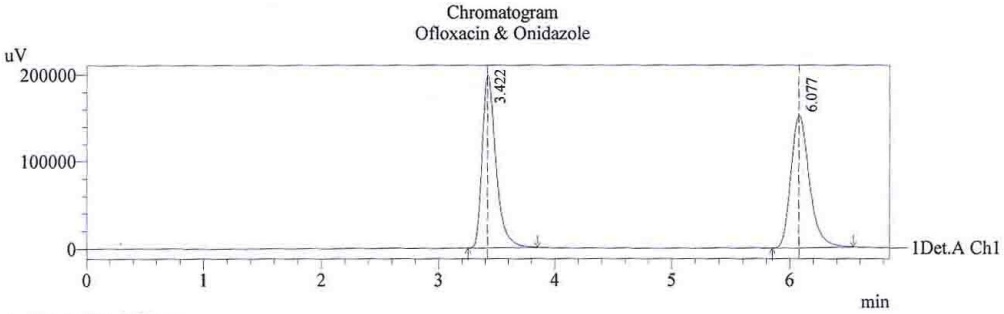
INTERMEDIATE PRECISION SAMPLE

Sample Information

Sample Name	: Intermediate Precision
Sample ID	: Sample Preparation 02
Tray#	: 1
Vail#	: 6
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1622429	198809	49.557	56.700
2	6.077	1651443	151822	50.443	43.300
Total		3273872	350631	100.000	100.000

PeakTable

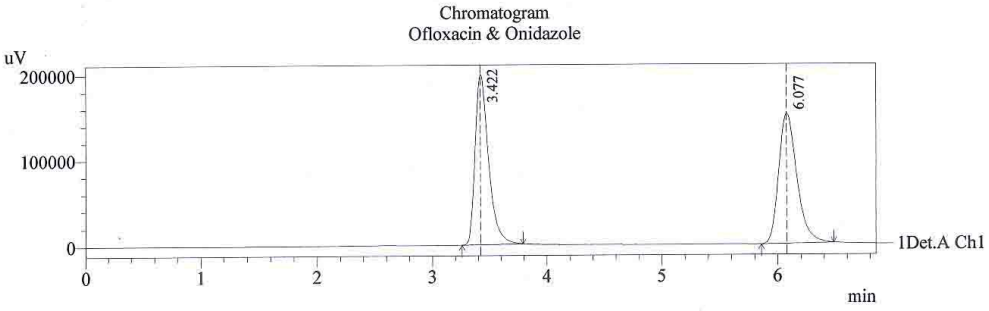
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1625190	198855	49.546	56.697
2	6.077	1654992	151881	50.454	43.303
Total		3280182	350735	100.000	100.000

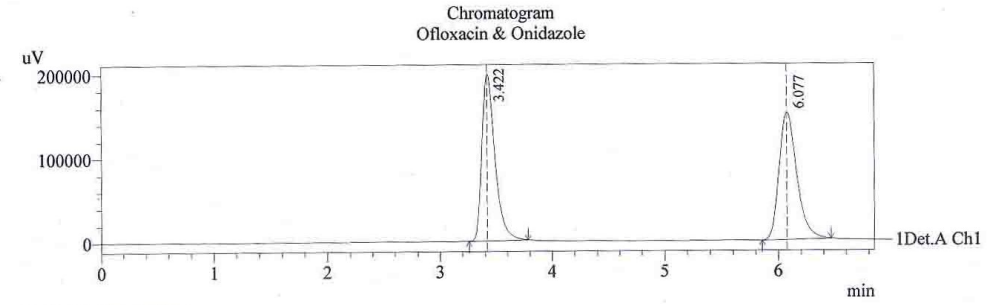
NTERMEDIATE PRECISION SAMPLE

Sample Information

Sample Name	: Intermediate Precision
Sample ID	: Sample Preparation 03
Tray#	: 1
Vail#	: 7
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1613362	198628	49.589	56.713
2	6.077	1640087	151604	50.411	43.287
Total		3253449	350232	100.000	100.000

PeakTable

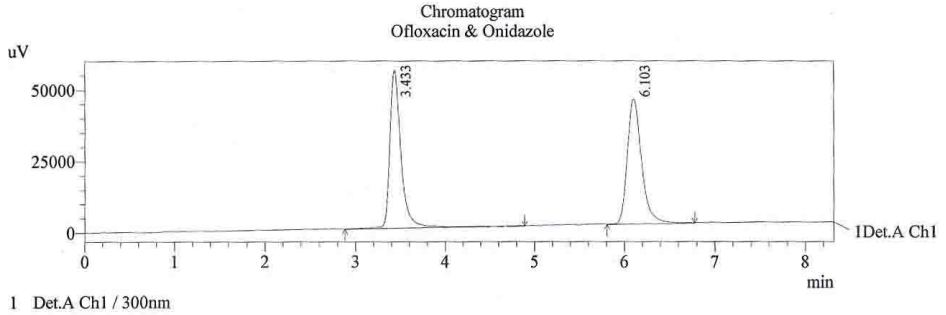
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1611356	198588	49.635	56.724
2	6.077	1635024	151506	50.365	43.276
Total		3246381	350094	100.000	100.000

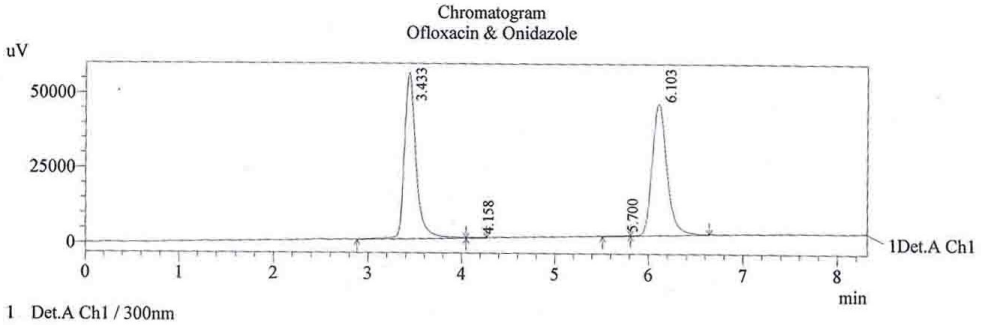
LINEARITY STANDARD

Sample Information

Sample Name	: Linearity Std Conc.
Sample ID	: Assay Linesrity
Tray#	: 1
Vail#	: 30
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.433	478739	55305	49.959	55.799
2	6.103	479517	43811	50.041	44.201
Total		958257	99116	100.000	100.000

PeakTable

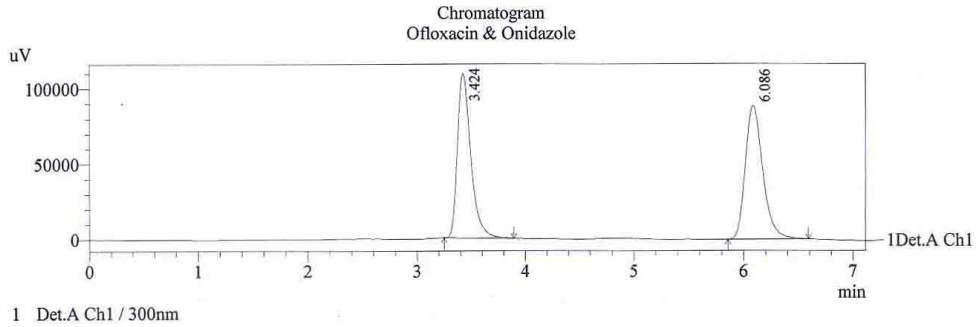
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.433	478306	55314	49.960	55.717
2	4.158	1099	99	0.115	0.100
3	5.700	1092	92	0.114	0.093
4	6.103	476887	43772	49.811	44.090
Total		957384	99277	100.000	100.000

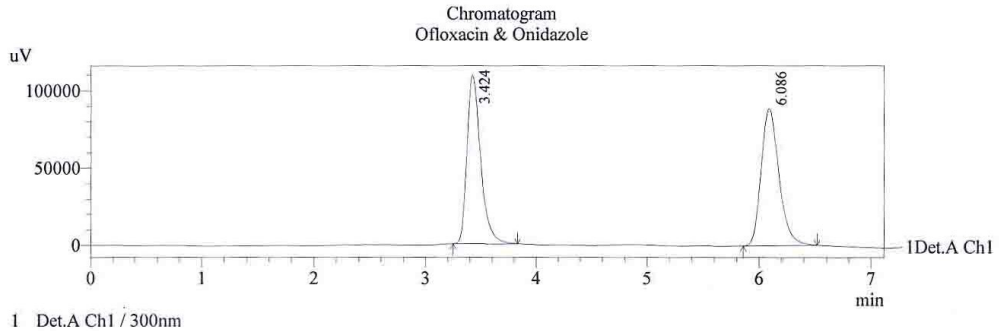
LINEARITY STANDARD

Sample Information

Sample Name	: Linearity Std Cond
Sample ID	: AssayLinearity
Tray#	: 1
Vail#	: 31
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	936507	108979	48.807	55.140
2	6.086	982284	88662	51.193	44.860
Total		1918791	197641	100.000	100.000

PeakTable

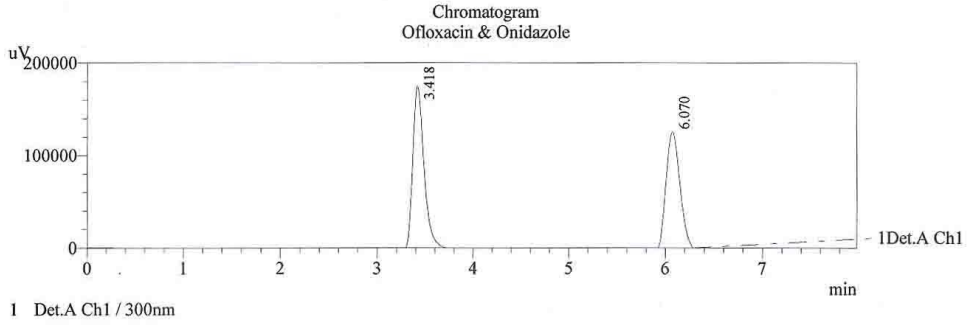
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	931496	108901	48.901	55.162
2	6.086	973366	88520	51.099	44.838
Total		1904861	197421	100.000	100.000

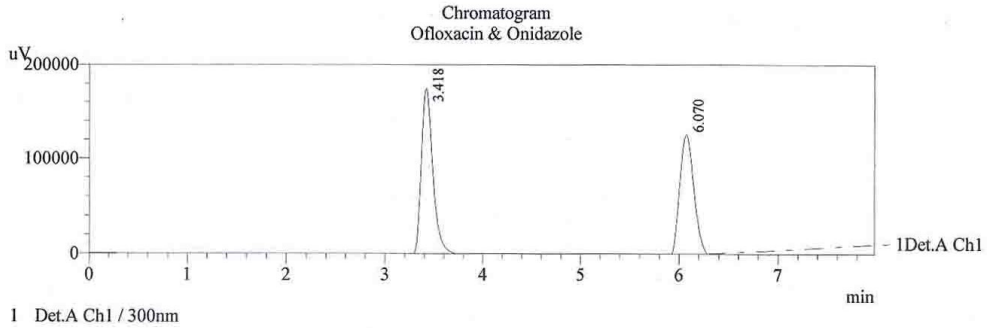
LINEARITY STANDARD

Sample Information

Sample Name	: Linearity Std Conc
Sample ID	: Assay Linearity
Tray#	: 1
Vail#	: 32
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.418	1432153	175715	49.825	56.694
2	6.070	1442213	134219	50.175	43.306
Total		2874367	309934	100.000	100.000

PeakTable

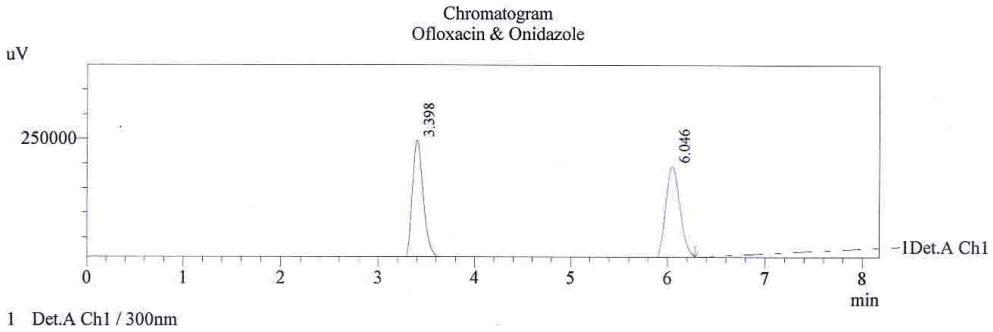
Detector A Ch1 300nm

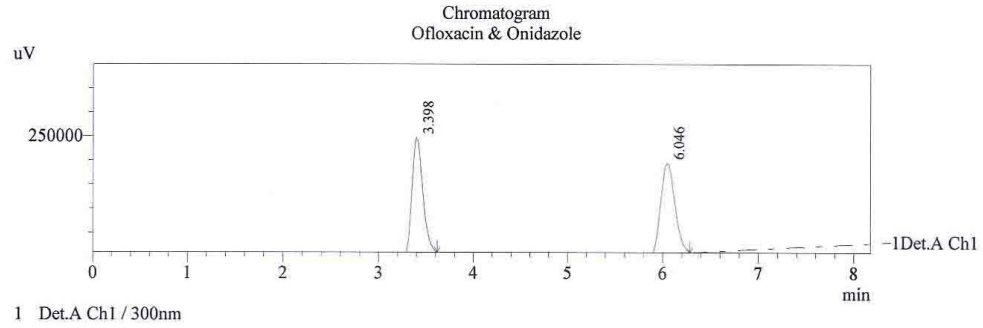
Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.418	1429294	175648	49.836	56.700
2	6.070	1438700	134138	50.164	43.300
Total		2867994	309786	100.000	100.000

LINEARITY STANDARD

Sample Information

Sample Name	: Linearity Std Conc
Sample ID	: Assay Linearity
Tray#	: 1
Vail#	: 33
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr





PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.398	1938199	242285	50.326	56.418
2	6.046	1913053	187161	49.674	43.582
Total		3851252	429446	100.000	100.000

PeakTable

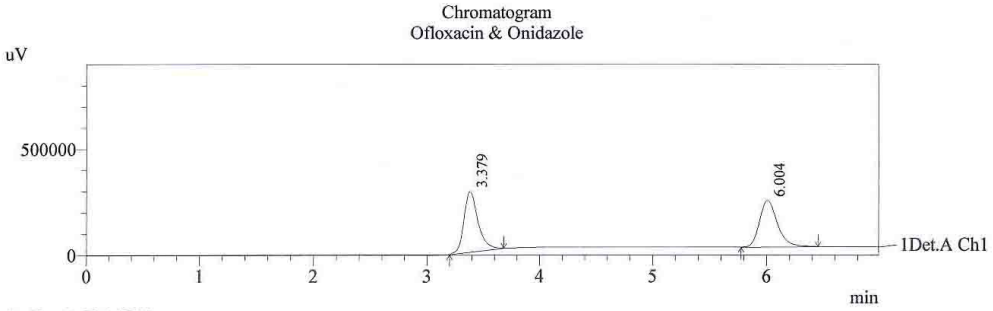
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.398	1924976	241809	50.155	56.370
2	6.046	1913053	187161	49.845	43.630
Total		3838029	428970	100.000	100.000

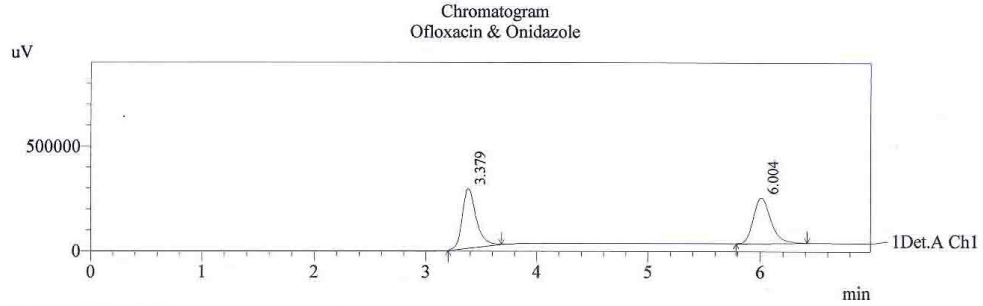
LINEARITY STANDARD

Sample Information

Sample Name	: Linearity Std Conc.
Sample ID	: Assay Linearity
Tray#	: 1
Vail#	: 34
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.379	2479720	285570	51.457	56.787
2	6.004	2339249	217311	48.543	43.213
Total		4818969	502881	100.000	100.000

PeakTable

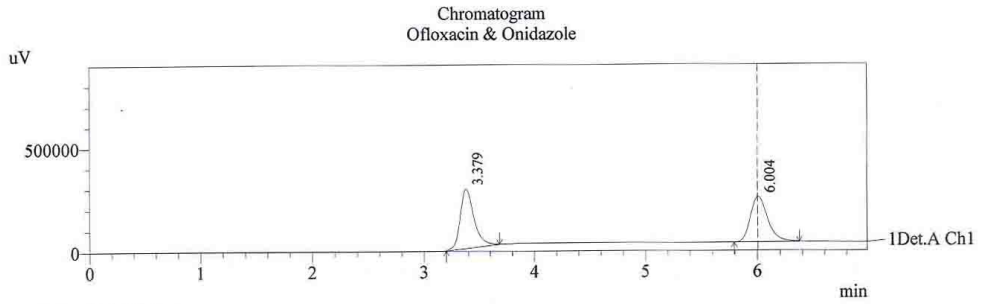
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.379	2479720	285570	51.584	56.813
2	6.004	2327417	217083	48.416	43.187
Total		4807137	502653	100.000	100.000

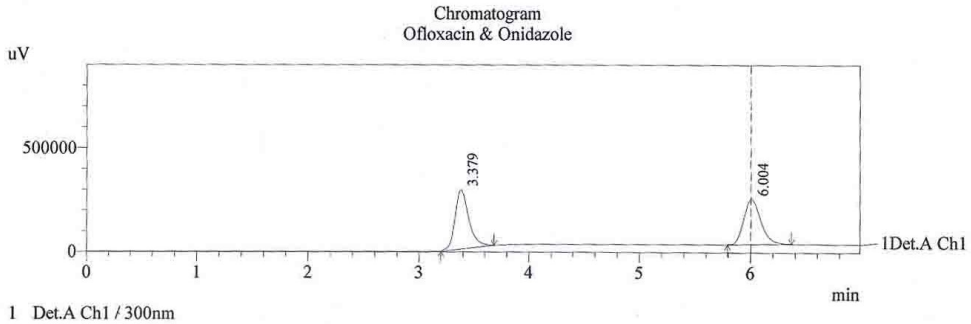
LINEARITY SAMPLE

Sample Information

Sample Name	: Linearity Sample Conc.
Sample ID	: Assay Linearity Sample 02
Tray#	: 1
Vail#	: 36
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.379	2479720	285570	51.799	56.860
2	6.004	2307462	216661	48.201	43.140
Total		4787183	502231	100.000	100.000

PeakTable

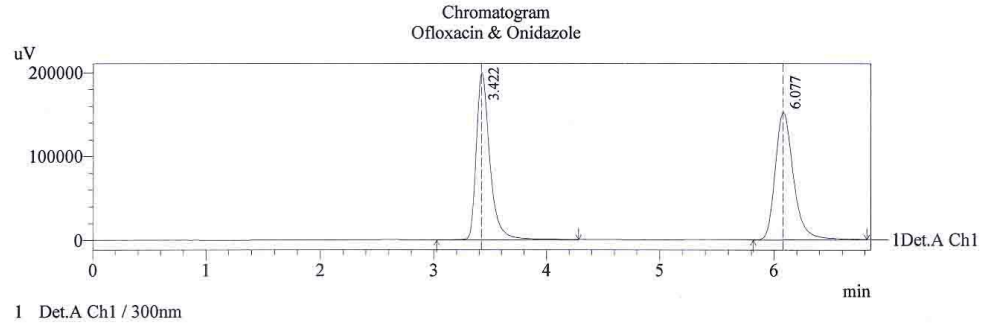
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.379	2479720	285570	51.847	56.871
2	6.004	2303069	216567	48.153	43.129
Total		4782789	502137	100.000	100.000

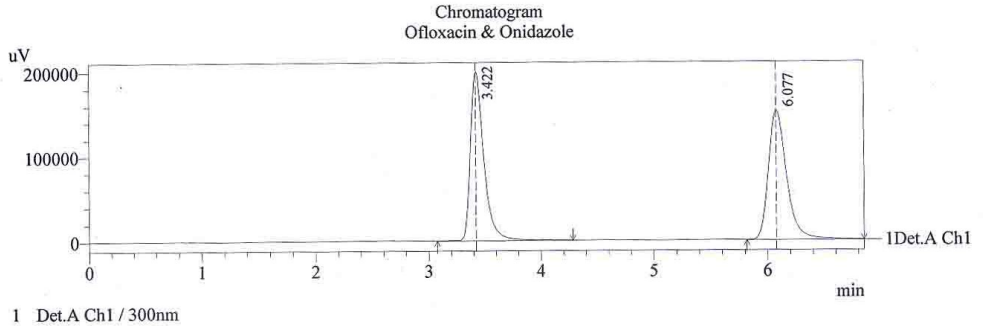
ACCURACY STANDARD

Sample Information

Sample Name	: Accuracy Std
Sample ID	: Assay Accuracy
Tray#	: 1
Vail#	: 1
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1666236	199521	49.700	56.710
2	6.077	1686376	152305	50.300	43.290
Total		3352612	351826	100.000	100.000

PeakTable

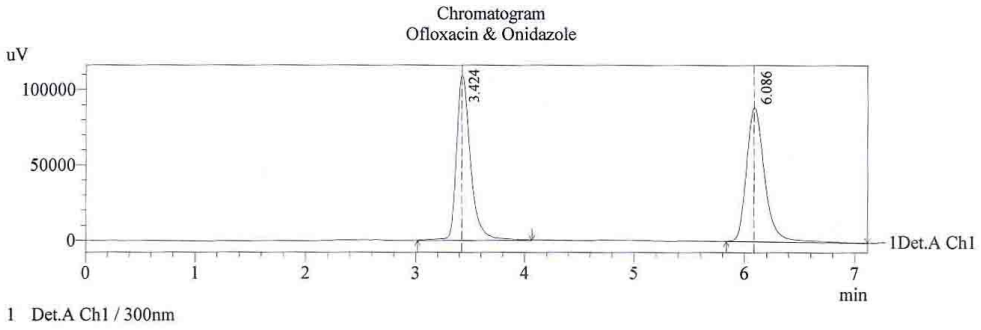
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1665510	199507	49.660	56.706
2	6.077	1688288	152321	50.340	43.294
Total		3353798	351828	100.000	100.000

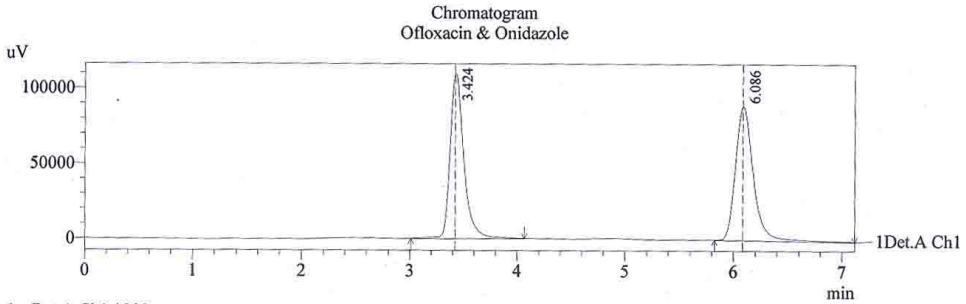
ACCURACY STANDARD

Sample Information

Sample Name	: Accuracy Std
Sample ID	: Assay Accuracy
Tray#	: 1
Vail#	: 03
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974531	109826	48.956	55.234
2	6.086	1016075	89010	51.044	44.766
Total		1990606	198835	100.000	100.000

PeakTable

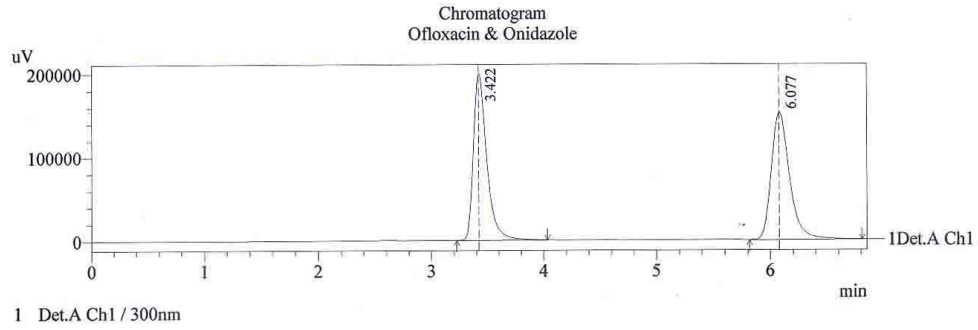
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974910	109833	48.927	55.227
2	6.086	1017674	89043	51.073	44.773
Total		1992584	198875	100.000	100.000

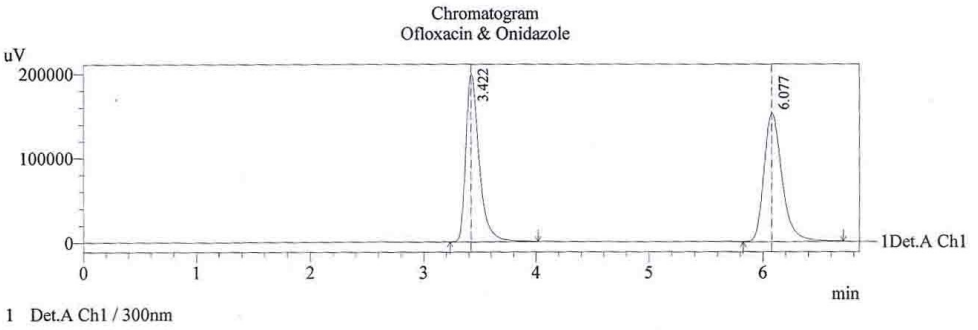
ACCURACY SAMPLE

Sample Information

Sample Name	: Accuracy Sample
Sample ID	: Assay Accuracy
Tray#	: 1
Vail#	: 2
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1643618	199151	49.366	56.665
2	6.077	1685834	152300	50.634	43.335
Total		3329451	351451	100.000	100.000

PeakTable

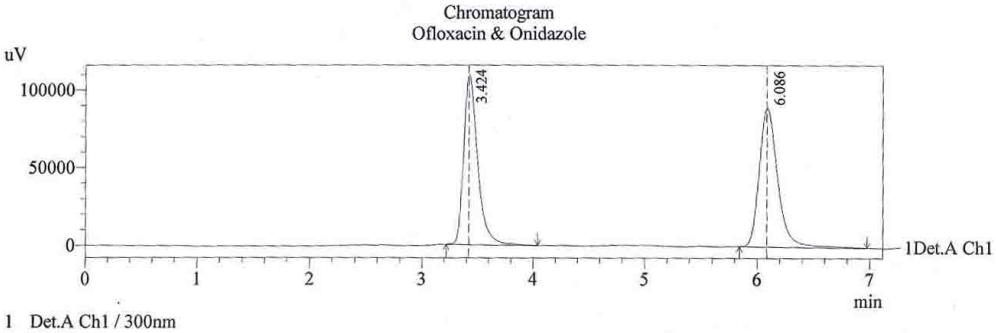
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.422	1641914	199116	49.456	56.674
2	6.077	1678036	152221	50.544	43.326
Total		3319950	351337	100.000	100.000

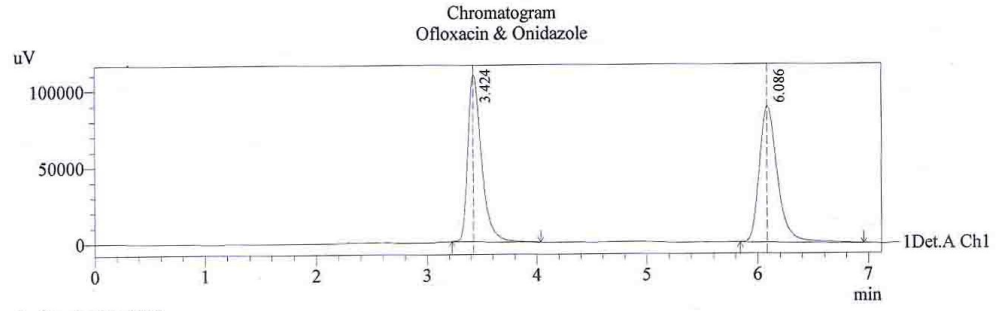
ACCURACY SAMPLE

Sample Information

Sample Name	: Accuracy Sample
Sample ID	: Assay Accuracy
Tray#	: 1
Vail#	: 04
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	949081	109238	48.466	55.118
2	6.086	1009170	88950	51.534	44.882
Total		1958251	198188	100.000	100.000

PeakTable

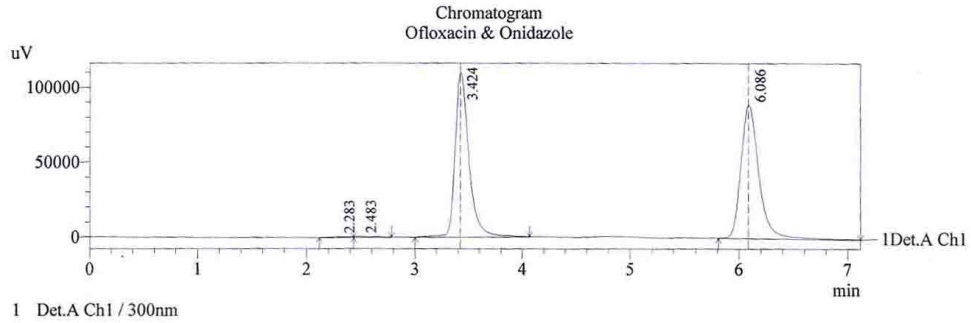
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	947761	109203	48.447	55.112
2	6.086	1008540	88946	51.553	44.888
Total		1956301	198149	100.000	100.000

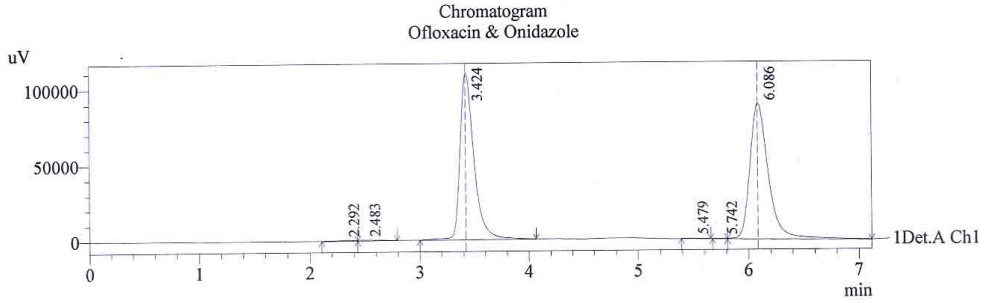
SOLUTION STABILITY STANDARD INITIAL

Sample Information

Sample Name	: Initial Solution Stability Std
Sample ID	: Assay Solution Stability
Tray#	: 1
Vail#	: 05
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.283	5027	327	0.250	0.164
2	2.483	6112	475	0.305	0.238
3	3.424	975223	109839	48.594	54.990
4	6.086	1020532	89101	50.851	44.608
Total		2006894	199742	100.000	100.000

PeakTable

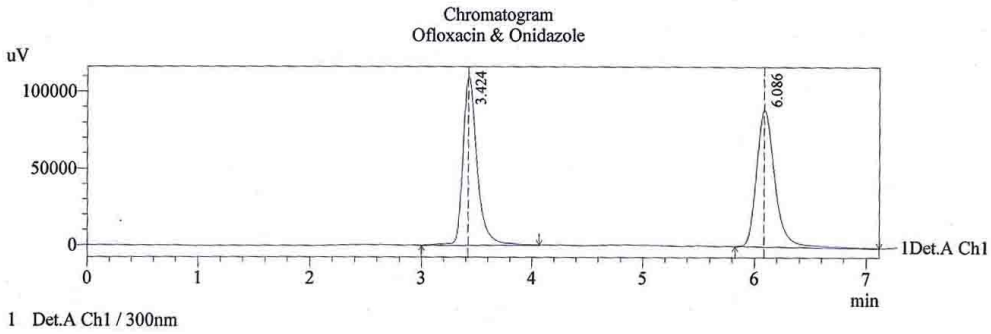
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.292	5166	340	0.256	0.170
2	2.483	6295	483	0.312	0.241
3	3.424	975223	109839	48.302	54.820
4	5.479	2094	251	0.104	0.125
5	5.742	1188	177	0.059	0.088
6	6.086	1029027	89271	50.967	44.555
Total		2018993	200362	100.000	100.000

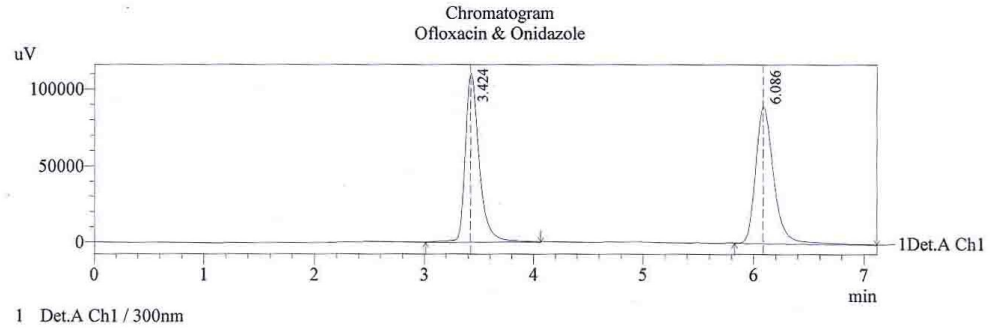
SOLUTION STABILITY STANDARD INITIAL

Sample Information

Sample Name	: Initial Solution Stability Sample
Sample ID	: Assay Solution Stability
Tray#	: 1
Vail#	: 06
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	975223	109839	48.935	55.228
2	6.086	1017674	89043	51.065	44.772
Total		1992897	198881	100.000	100.000

PeakTable

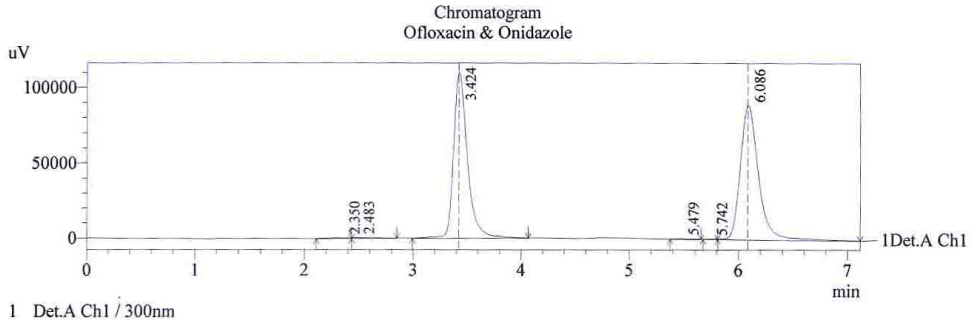
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974910	109833	48.927	55.227
2	6.086	1017674	89043	51.073	44.773
Total		1992584	198875	100.000	100.000

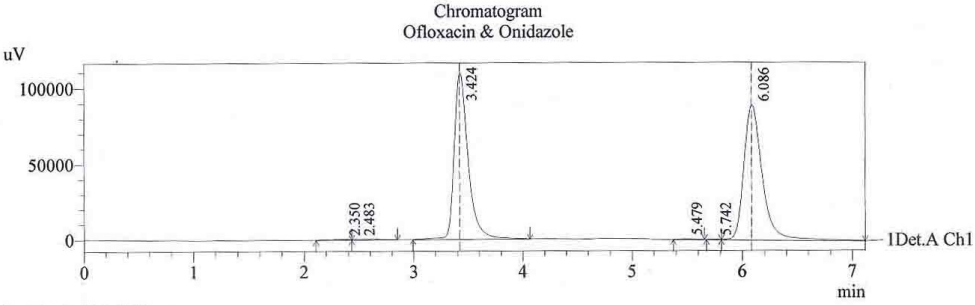
SOLUTION STABILITY STANDARD - 12 HOURS

Sample Information

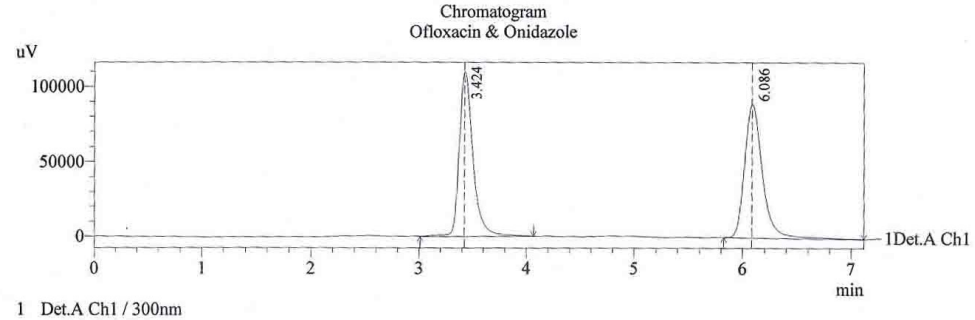
Sample Name	: 12hr Solution Stability Std
Sample ID	: Assay Solution Stability
Tray#	: 1
Vail#	: 05
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



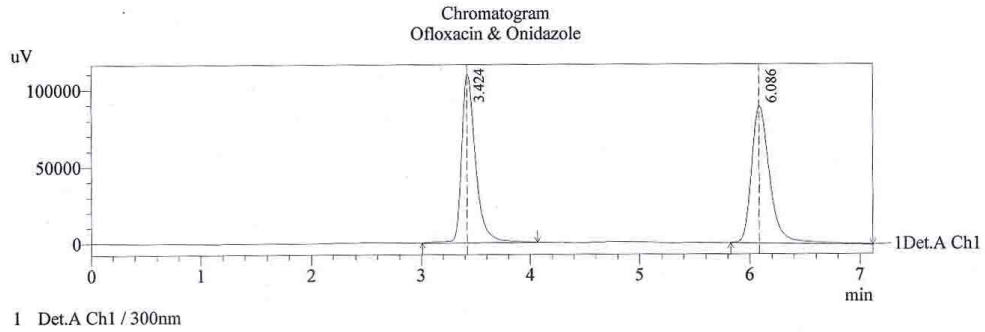
1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.350	5484	378	0.271	0.189
2	2.483	7486	521	0.370	0.260
3	3.424	975421	109842	48.265	54.795
4	5.479	2354	271	0.116	0.135
5	5.742	1188	177	0.059	0.088
6	6.086	1029027	89271	50.918	44.533
Total		2020961	200461	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.350	5484	378	0.271	0.189
2	2.483	7486	521	0.370	0.260
3	3.424	975421	109842	48.265	54.795
4	5.479	2354	271	0.116	0.135
5	5.742	1188	177	0.059	0.088
6	6.086	1029027	89271	50.918	44.533
Total		2020961	200461	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974910	109833	48.927	55.227
2	6.086	1017674	89043	51.073	44.773
Total		1992584	198875	100.000	100.000

PeakTable

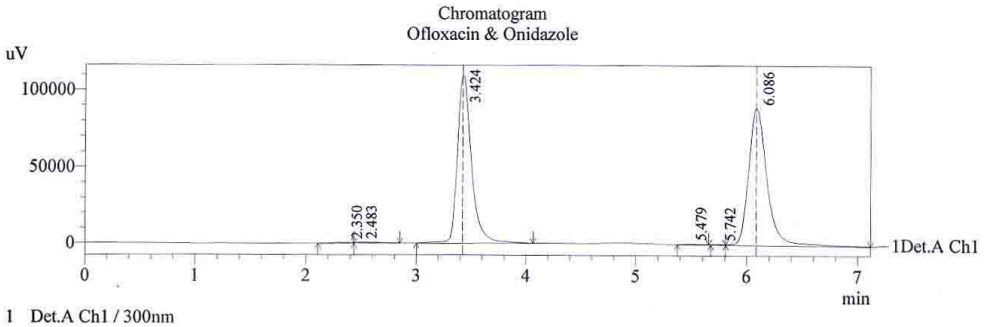
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974910	109833	48.927	55.227
2	6.086	1017674	89043	51.073	44.773
Total		1992584	198875	100.000	100.000

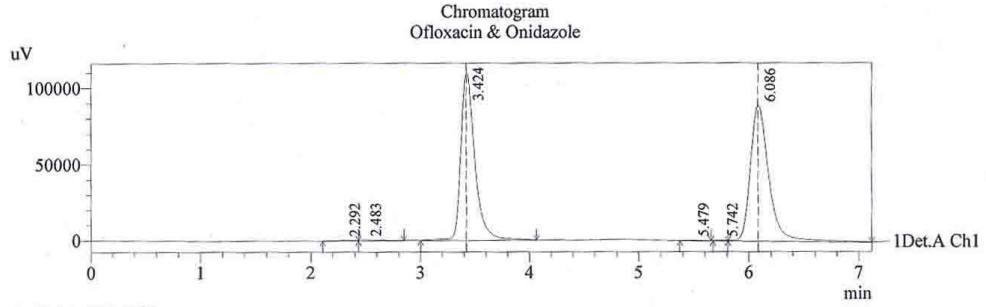
SOLUTION STABILITY STANDARD - 24 HOURS

Sample Information

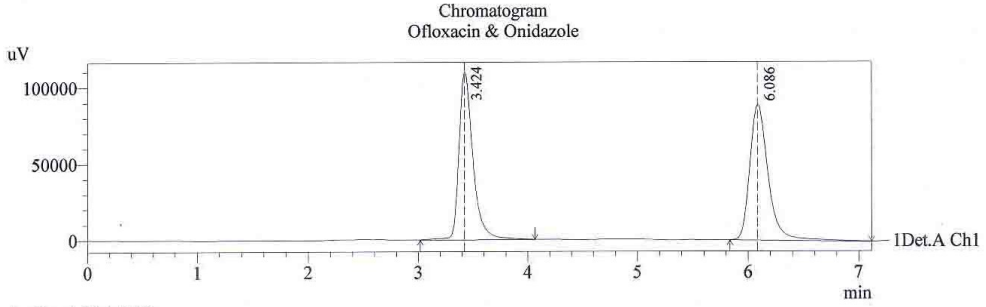
Sample Name	: 24hr Solution Stability Std
Sample ID	: Assay Solution Stability
Tray#	: 1
Vail#	: 05
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



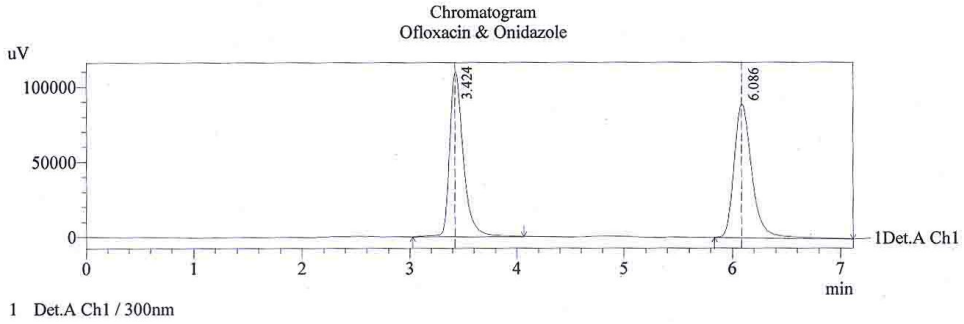
1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.350	5484	378	0.271	0.189
2	2.483	7486	521	0.370	0.260
3	3.424	975223	109839	48.260	54.794
4	5.479	2354	271	0.116	0.135
5	5.742	1188	177	0.059	0.088
6	6.086	1029027	89271	50.923	44.534
Total		2020763	200458	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.292	5484	359	0.271	0.179
2	2.483	7486	521	0.370	0.260
3	3.424	975223	109839	48.260	54.799
4	5.479	2354	271	0.116	0.135
5	5.742	1188	177	0.059	0.088
6	6.086	1029027	89271	50.923	44.538
Total		2020763	200438	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974531	109826	48.956	55.234
2	6.086	1016075	89010	51.044	44.766
Total		1990606	198835	100.000	100.000

PeakTable

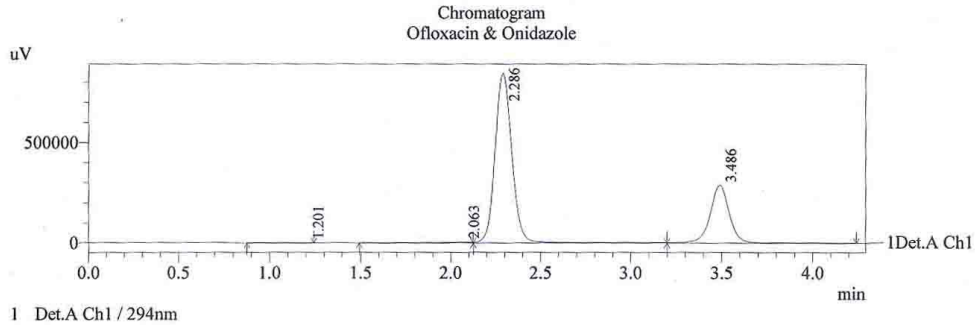
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	974289	109821	48.950	55.233
2	6.086	1016075	89010	51.050	44.767
Total		1990364	198831	100.000	100.000

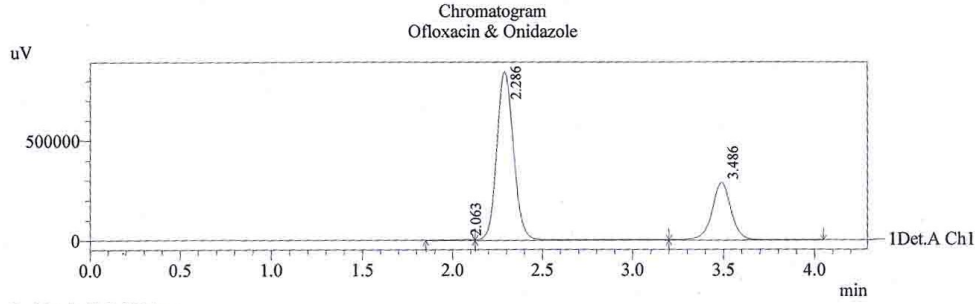
ROBUSTNESS STANDARD

Sample Information

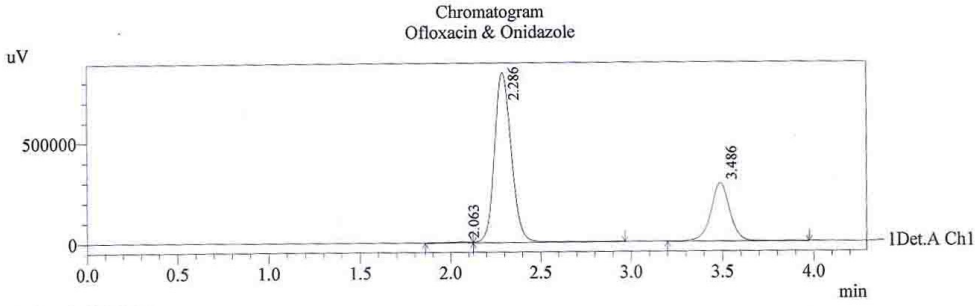
Sample Name	: Robustness Std
Sample ID	: Std
Tray#	: 1
Vail#	: 1
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 294nm



1 Det.A Ch1 / 294nm



1 Det.A Ch1 / 294nm

PeakTable

Detector A Ch1 294nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	1.201	1243	97	0.016	0.009
2	2.063	22444	3245	0.298	0.286
3	2.286	5451218	845398	72.302	74.411
4	3.486	2064640	287382	27.384	25.295
Total		7539546	1136122	100.000	100.000

PeakTable

Detector A Ch1 294nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.063	21102	3195	0.280	0.281
2	2.286	5448009	845348	72.342	74.423
3	3.486	2061778	287332	27.378	25.296
Total		7530889	1135875	100.000	100.000

PeakTable

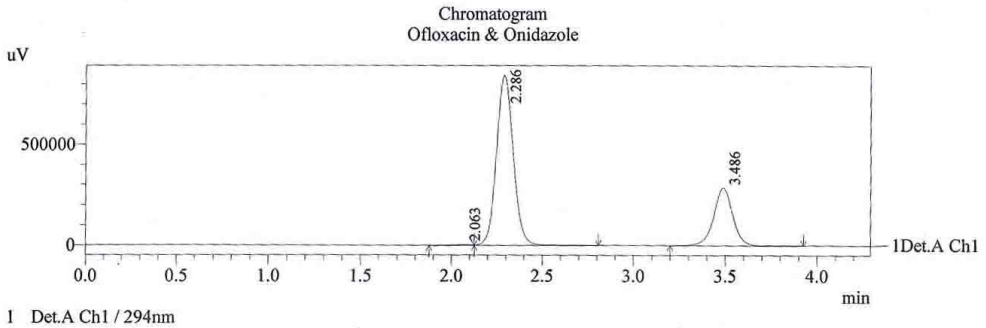
Detector A Ch1 294nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.063	20413	3131	0.272	0.276
2	2.286	5434137	845218	72.342	74.432
3	3.486	2057174	287215	27.386	25.293
Total		7511724	1135564	100.000	100.000

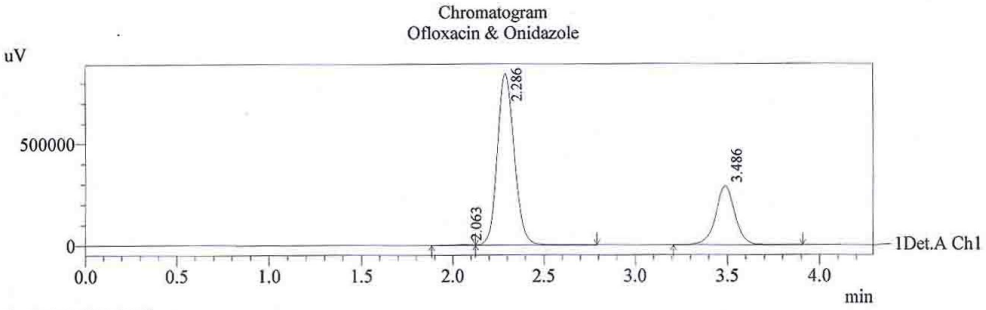
ROBUSTNESS SAMPLE

Sample Information

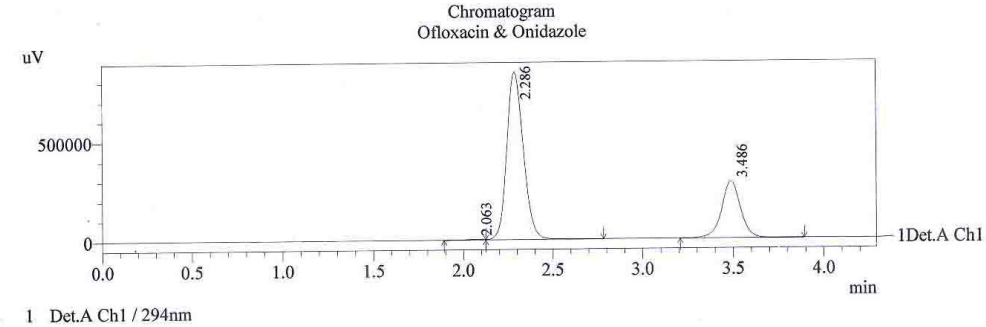
Sample Name	: Robustness Sample
Sample ID	: Sample
Tray#	: 1
Vail#	: 2
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 294nm



1 Det.A Ch1 / 294nm



1 Det.A Ch1 / 294nm

PeakTable

Detector A Ch1 294nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.063	19936	3087	0.266	0.272
2	2.286	5428058	845129	72.328	74.433
3	3.486	2056797	287209	27.406	25.295
Total		7504792	1135425	100.000	100.000

PeakTable

Detector A Ch1 294nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.063	19853	3081	0.265	0.271
2	2.286	5427487	845118	72.328	74.433
3	3.486	2056623	287205	27.407	25.295
Total		7503962	1135404	100.000	100.000

PeakTable

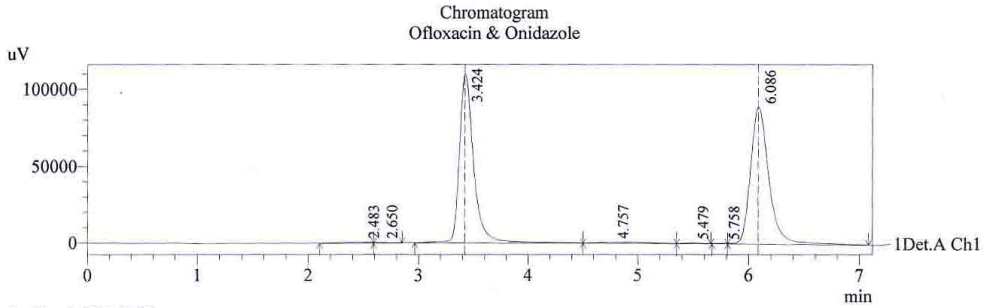
Detector A Ch1 294nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.063	19752	3073	0.263	0.271
2	2.286	5427108	845110	72.330	74.434
3	3.486	2056356	287200	27.406	25.295
Total		7503215	1135383	100.000	100.000

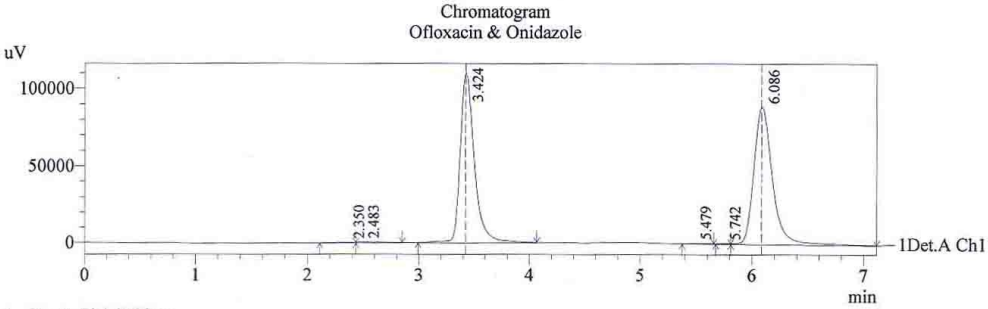
ROBUSTNESS STANDARD

Sample Information

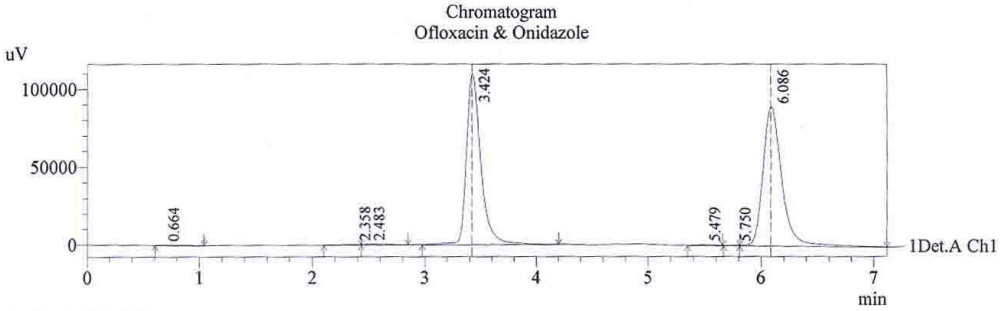
Sample Name	: Robustness Std
Sample ID	: Std
Tray#	: 1
Vail#	: 07
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.483	9950	523	0.485	0.260
2	2.650	3112	393	0.152	0.195
3	3.424	989622	109978	48.231	54.636
4	4.757	17417	602	0.849	0.299
5	5.479	2737	297	0.133	0.148
6	5.758	1313	226	0.064	0.113
7	6.086	1027677	89270	50.086	44.349
Total		2051828	201290	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	2.350	5484	378	0.271	0.189
2	2.483	7486	521	0.370	0.260
3	3.424	975421	109842	48.265	54.795
4	5.479	2354	271	0.116	0.135
5	5.742	1188	177	0.059	0.088
6	6.086	1029027	89271	50.918	44.533
Total		2020961	200461	100.000	100.000

PeakTable

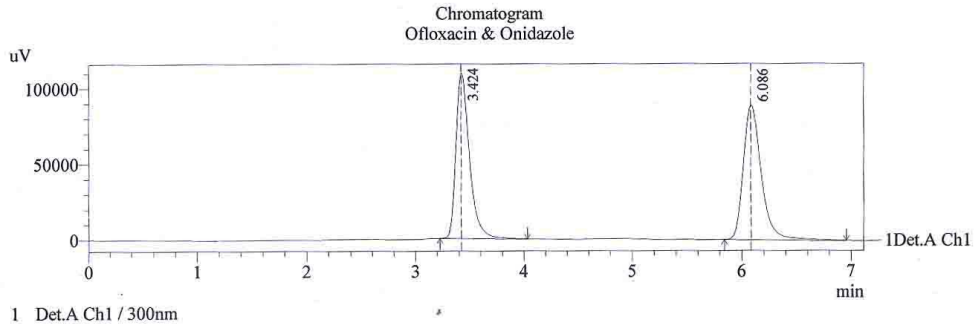
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	0.664	2580	135	0.127	0.067
2	2.358	5548	386	0.273	0.192
3	2.483	7514	523	0.370	0.261
4	3.424	980784	109904	48.311	54.748
5	5.479	2737	297	0.135	0.148
6	5.750	1333	215	0.066	0.107
7	6.086	1029666	89284	50.718	44.477
Total		2030161	200744	100.000	100.000

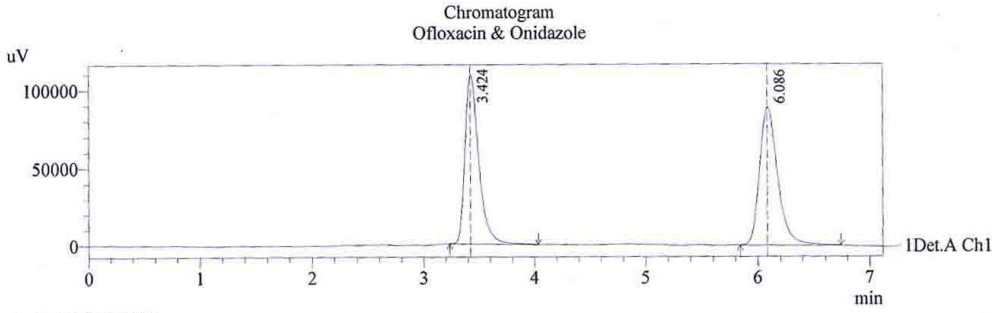
ROBUSTNESS SAMPLE

Sample Information

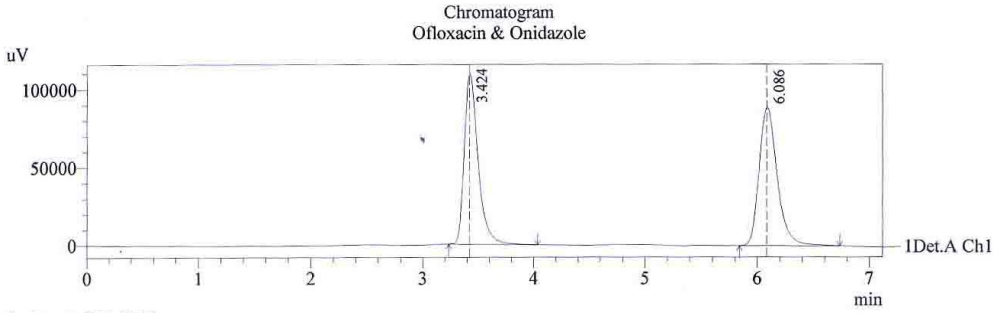
Sample Name	: Robustness Sample
Sample ID	: Sample
Tray#	: 1
Vail#	: 08
Injection Volume	: 20 uL
Data Filename	: Ofloxacin & Onidazole.lcm01.lcd
Method Filename	: Ofloxacin & Onidazole.lcm
Batch Filename	:
Report Filename	: Default.lcr



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm



1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	947761	109203	48.447	55.112
2	6.086	1008540	88946	51.553	44.888
Total		1956301	198149	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	946494	109164	48.765	55.135
2	6.086	994424	88829	51.235	44.865
Total		1940918	197992	100.000	100.000

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.424	946494	109164	48.776	55.136
2	6.086	993988	88824	51.224	44.864
Total		1940482	197988	100.000	100.000

Chapter – 7

Results & Discussion

7. RESULTS AND DISCUSSION

The working condition for the HPLC established for Ofloxacin and Ornidazole and then was applied on pharmaceutical dosage forms (a combination of Ofloxacin and Ornidazole tablet was used) . A simple reverse phase High Performance Liquid Chromatography has been developed and subsequently validated.

The separation method was carried out by using a mobile phase consisting of Potassium dihydrogen phosphate buffer (0.05M): (Acetonitrile and Tetrahydrofuran 35:1v/v) pH 3.5 ± 0.1 the ratio of 64:35 v/v.

The deduction was carried out by using UV detector at 300nm. The column was Agilent C 18 (250X4.6mm) 5µ. The flow rate was selected as 1.0ml/min.

The retention time of Ofloxacin and Ornidazole was found to be 4.22 & 6.07. The number of theoretical plates of Ofloxacin and Ornidazole was found to be 4518.60 & 7508.51 which indicates the efficient performance of the column. These parameters represent the specificity of the method.

From the linearity studies, specified concentration levels were determined. It was observed that Ofloxacin and Ornidazole were linear in the range of 33.33% to 166.66% for the target concentration by RP-HPLC. The linearity range of Ofloxacin and Ornidazole 33.33% to 166.66% µg/ml was found to obey linearity with a correlation coefficient (r^2) of 1.012 & 1.110 respectively.

The validation of the proposed method was verified by system precision and method precision by RP-HPLC.

The validation of the proposed method was verified by recovery studies. The percentage recovery range was found to be satisfied which represent in results. The ruggedness study was also performed. The robustness studies were performed.

The validation of the proposed method was verified by system suitability. The % RSD of the system suitability for Ofloxacin and Ornidazole was found to be 0.447 & 1.120.

The validation of the proposed method was verified by precision, recovery studies and solution stability. The percentage of precision, recovery studies and solution stability was found to be satisfied which represent in results. These are all comes under the specified limits and passes.

The analytical method validation was carried out by RP-HPLC as per ICH guidelines which are mentioned below as follows.

METHOD VALIDATION REPORT BY RP – HPLC

S.No	PARA-METERS	LIMIT	OBSERVATIONS	PASSES/ FAILS
1	Specificity	No Interferences at retention time of the peak.	No Interference at retention time of the peak	Passes
2	Linearity	Correlation co-efficient (r^2) NLT 0.999	Ofloxacin -1.012 Ornidazole - 1.110	Passes
3.	Precision	%RSD NMT 2.0	Ofloxacin -1.630 Ornidazole -0.982	Passes
4	Accuracy	% Recovery range 98-102%	Within limits	Passes
5.	Ruggedness	RSD NMT 2.0%	Within limits	Passes
6.	Robustness	%RSD NMT 2.0	Within limits	Passes

Chapter – 8

Summary & Conclusion

8. SUMMARY AND CONCLUSION

A HPLC for Ofloxacin and Ornidazole was developed and validated in combined tablet dosage form as pre ICH Guide lines

UV Detector and Agilent C $_{18}$ (250x4.6mm) 5µ column, injection of 20µl is injected and eluted with the mobile phase of Potassium dihydrogen phosphate buffer with pH3.5: (Acetonitrile & Tetra hydro furan35:1 v/v) in the ratio 64.9:35 which was pumped at a flow rate of 1.0ml at 300nm. The peak of Within limits was found well separated within **8min**. The developed method was validated for various parameters as per ICH guidelines like system suitability, specificity, linearity, system precision, method precision, accuracy, ruggedness and robustness.

The analytical method validation of Ofloxacin and Ornidazole by RP-HPLC was found to be satisfactory and could be used for the routine pharmaceutical analysis of Ofloxacin and Ornidazole.

Chapter – 9

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